Tampere University

KRISTINA VESKIMÄE

High Grade Serous Ovarian Cancer

Expression Profiling, Aspects of Early Pathogenesis and Potential Mechanisms of Chemoresistance

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ACADEMIC DISSERTATION
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ACADEMIC DISSERTATION
Tampere University, Faculty of Medicine and Health Technology
Tampere University Hospital, Department of Obstetrics and Gynecology
Finland

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Start by doing what is necessary, then what is possible, and suddenly you are doing the impossible.

— St. Francis Of Assisi

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Tampere, November 2019

Kristina Veskimäe

ABSTRACT

Ovarian cancer is the 5th most common malignancy in European women, but it occupies 1st place in the mortality statistics for gynecological malignancies. In Finland, approximately 500 women are diagnosed yearly, and over 200 women die of the disease each year. There is a need for research in this field to better understand the pathogenesis of the disease, as well as to develop treatments.

In this dissertation, the early pathogenesis of epithelial ovarian cancer (EOC) was investigated by comparing the genome-wide gene expression levels in *BRCA1/2* -mutation-positive risk-reducing salpingo-oophorectomy (RRSO) samples to those in healthy controls. The study revealed differentially expressed genes by microarray analysis, from which selected genes were validated by quantitative real time polymerase chain reaction (qRT-PCR), demonstrating comparable expression patterns between *BRCA1/2*-mutation-positive RRSO and high-grade serous ovarian cancer (HGSC) samples.

In addition, in light of recent vivid research in the field of targeted therapy, specifically that of Poly(ADP)Ribose Polymerase (PARP) inhibitors in EOC, PARP expression was investigated. A PARP pharmacodynamic assay revealed an association between high PARP activity and platinum sensitivity and longer progression-free survival (PFS), which is a novel finding. Furthermore, neoadjuvant chemotherapy (NACT) seemed to be associated with low PARP activity. PARP immunohistochemistry (IHC) staining and enzyme-linked immunosorbent assay (ELISA) pharmacodynamic assay PARP activity measurements were not associated, and the PARP pharmacodynamic assay may reflect more biologically significant PARP relative to PARP IHC.

Third, exploring chemoresistance in EOC was undertaken, yielding a novel result showing the association of differential expression of *ROR2* and *GREB1* with treatment response in HGSC. In detail, the Wnt5a/ROR2 pathway was found to be potentially actionable in the possible modulation of chemoresistance in EOC. A combination of ROR antagonists and chemotherapeutic agents may be an investigation-worthy option in the future, as silencing ROR1 and ROR2 restores the chemosensitivity of carboplatin-resistant ovarian cancer cells.

TIIVISTELMÄ

Munasarjasyöpä on viidenneksi yleisin gynekologinen syöpä eurooppalaisessa väestössä, mutta gynekologisten syöpäkuolemien tilastoissa se on ensimmäisellä paikalla. Suomessa noin 500 naista vuodessa saa munasarjasyöpädiagnoosin, siihen liittyviä kuolemia on kuitenkin yli 200. Tämän vuoksi munasarjasyövän varhaisen patogeneesin selvittely ja hoitoihin liittyvä tutkimus on tärkeässä roolissa.

Väitöstutkimuksessa selvitettiin epiteliaalisen munasarjasyövän varhaista kehitystä vertailemalla *BRCA 1/2* -mutaationkantajien morfologisesti normaaleja munasarja- ja munatorvinäytteitä hyvänlaatuisen kohtuperäisen syyn vuoksi poistettuihin munasarja- ja munatorvinäytteisiin mikrosirumenetelmällä. Tässä tutkimuksessa havaitut *BRCA1/2*-mutaationäytteissä merkitsevästi erilailla ilmentyneet geenit saattavat olla merkityksellisiä munasarjasyövän varhaisessa kehityksessä.

Lisäksi tutkittiin PARP:n aktiivisuutta ELISA-menetelmällä ja sen ilmentymistä immunohistokemiallisella (IHC) menetelmällä high-grade munasarjasyöpäpotilaiden kohortissa. PARP-estäjät ovat ns. uusi räätälöidyn hoidon lääkeryhmä. Tässä tutkimuksessa todettiin, että PARP-aktiivisuus on yhteydessä platinaherkkyyteen ja pidempään tautivapaaseen aikaan. Lisäksi neoadjuvanttihoidon todettiin olevan yhteydessä matalaan PARP-aktiivisuuteen. ELISA-menetelmällä mitattu PARP-aktiivisuus ja IHC-värjäyksillä tutkittu PARP ilmentymä eivät korreloineet keskenään; PARP on mahdollisesti paremmin osoitettavissa tuorenäytteistä aktiivisuustutkimuksena verrattuna parafiiniblokeista väräyksillä saatuihin tuloksiin.

Väitöstutkimuksessa tarkasteltiin lisäksi myös platinavasteeseen liittyviä tekijöitä samassa high-grade munasarjasyöpäpotilaskohortissa. Tutkimuksessa havaittiin yhteys *ROR2*- ja *GREB1*- ilmentymän ja platinaherkkyyden välillä. Lisäksi Wnt5a/ROR2 signalointireitti vaikutti mahdolliselta muokkauksen kohteelta lääkeresistenssin modulaatiossa. Aiempien tutkimusten perusteella ROR1 ja ROR2 reseptorien hiljentäminen platinaresistenteillä munasarjasyöpäsoluilla palauttaa kemosensitiivisyyden näissä soluissa ja näin ollen tulevaisuudessa ROR-antagonistin ja solusalpaajan yhdistelmä voisi olla lupaava vaihtoehto jatkotutkimuksia ajatellen.

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ABBREVIATIONS

AGO	Arbeitsgruppe der Gynäkologischer Onkologie
ALDH	aldehyde dehydrogenase
AUC	area under the curve
BCL2	B-cell lymphoma-2
BMI	body mass index
BOT	borderline tumor
BRCA	BReast CAncer (susceptibility gene)
CA 125	cancer antigen 12-5, carbohydrate antigen 12-5
CI	confidence interval
CPLD	liposomal doxorubicin
CT	computed tomography
d.e.	differentially expressed
DNA	DeoxyriboNucleic Acid
DDR	DNA damage repair
DSB	double strand break
ELISA	enzyme-linked immunosorbent assay
EMEA	European Agency for the Evaluation of Medicinal Products
EOC	epithelial ovarian cancer
EORTC	European Organization for Research and Treatment of Cancer
ER	estrogen receptor
FDA	Food and Drug Association
FIGO	International Federation of Gynecology and Obstetrics
GEO	Gene Expression Omnibus
GO	gene ontology
HE4	human epididymis protein 4
HGSC	High Grade serous ovarian cancer

HIPEC	hyperthermic intraoperative intraperitoneal chemotherapy
HNPCC	hereditary non-polyposis colorectal cancer
HR	hazard ratio
HR	homologous recombination
HRD	homologous recombination deficiency
HRT	hormone replacement therapy
IHC	immunohistochemistry
IDS	interval debulking surgery
IGF-1	insulin-like growth factor 1
IP	intraperitoneal
IU	international unit
K-ras	Kirsten-rat sarcoma viral oncogene homolog
LGSC	Low Grade serous ovarian cancer
LND	lymphadenectomy
LNG-IUS	levonorgestrel-releasing intrauterine system
LOH	loss of heterozygosity
MDR	multiple drug resistance
MMR	mismatch repair
miRNA	microRNA
MRI	magnetic resonance imaging
mRNA	messenger RNA
NACT	neoadjuvant chemotherapy
NGS	next-generation sequencing
NHEJ	non-homologous-end-joining
NPV	negative predictive value
NSGO	Nordic Society of Gynecologic Oncology
ncRNA	non-coding RNA
OC	ovarian cancer
OR	odds ratio
OS	overall survival
qRT-PCR	quantitative real time polymerase chain reaction
PARP	Poly(ADP)Ribose Polymerase
PARPi	PARP inhibitor
PAX8	paired box gene 8

PBMCOC	patients with BRCA1/2 mutation carrier ovarian cancer
p53	tumor suppressor protein 53
PCOS	polycystic ovarian syndrome
PCR	polymerase chain-reaction
PDS	primary debulking surgery
PET	positron emission tomography
PFI	platinum free interval
PFS	progression free survival
PID	pelvic inflammatory disease
PPV	positive predictive value
PR	progesterone receptor
PTEN	phosphatase and tensin homolog
RCT	randomized controlled trial
RMI	risk of malignancy index
RNA	RiboNucleic Acid
ROMA	risk of ovarian malignancy algorithm
RR	relative risk
RRSO	risk-reducing salpingo-oophorectomy
Src	Rous sarcoma virus oncogene
qRT-PCR	quantitative, reverse transcription PCR
SD	standard deviation
SET	solid, pseudoendometroid, transitional carcinoma
SSB	single strand break
STIC	serous tubal intra-epithelial carcinoma
TAI	telomeric allelic imbalance
TAUH	Tampere University Hospital
TCGA	The Cancer Genome Atlas
TdT	terminal deoxynucleotidyl transferase
TFI	therapy free interval
TILT	tubal intraepithelial lesions in transition
TNFAIP8	tumor necrosis factor α-induced protein 8
TVU	transvaginal ultrasound
VEGF	vascular endothelial growth factor
vs	versus

ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

Publication I Veskimäe, K., Staff, S., Tabaro, F , Nykter, M. , Isola, J., Mäenpää, J. Microarray analysis of differentially expressed genes in ovarian and fallopian tube epithelium from risk-reducing salpingo-oophorectomies. Genes Chromosomes Cancer. 2015 May;54(5):276-87.

Publication II Veskimäe, K., Staff, S., Grönholm, A, Pesu, M., Laaksonen, M., Nykter, M., Isola, J. and Mäenpää, J. Assessment of PARP protein expression in epithelial ovarian cancer by ELISA pharmacodynamic assay and immunohistochemistry. Tumour Biol. 2016 Sep;37(9):11991-11999.

Publication III Veskimäe K*, Scaravilli M*, Ungureanu, D, Karvonen H, Niininen W, Jaatinen S, Nykter M, Isola J, Mäenpää J, Visakorpi T and Staff S. Expression analysis of platinum sensitive and resistant epithelial ovarian cancer patient samples reveals new biomarkers for targeted therapies. Transl Oncol. 2018 Oct;11(5):1160-1170.

* equal contribution

The publications are referred to in the text by their Roman numerals. The original publications have been reprinted here with the kind permission of their copyright holders.

1 INTRODUCTION

Ovarian cancer is an aggressive disease that is often diagnosed at a late stage and has a poor prognosis. The standard treatment consists of surgery and chemotherapy; many patients, however, relapse after completing primary treatment and eventually become resistant to chemotherapy (Rojas et al., 2016).

The early pathogenesis of ovarian cancer has been extensively investigated using modern molecular biology techniques. These efforts have produced an understanding that is now widely accepted, namely, that high-grade serous ovarian cancer (HGSC) in fact arises from the Fallopian tube (Kurman and Shih, 2010). The Fallopian tube theory has benefitted greatly from data extracted from BRCA mutation carriers' prophylactic salpingo-oophorectomy samples. Trailblazing work investigating the origin of HGSC has demonstrated that serous tubal intraepithelial carcinoma (STIC) is the precursor for HGSC (Prat, 2012). To emphasize, HGSC is currently viewed as an entity of three related diseases – high-grade epithelial Fallopian tube cancer, high-grade epithelial ovarian cancer and primary peritoneal cancer (Singh et al., 2017).

The challenge in ovarian cancer remains to be late diagnosis leading to poor prognosis. In this regard, molecular subtyping and detailed profiling might aid in earlier and more accurate diagnosis and perhaps screening. The work exploring Fallopian tubes and prophylactic salpingectomies has already provided an opportunity for prevention in high-risk patients.

Currently, the standard treatment for ovarian cancer entails surgery combined with 6 cycles of platinum-based chemotherapy in further combination with anti-angiogenetic therapy in high-risk patients (Reuss et al., 2019). Ovarian cancer (OC) is chemoresponsive, but not a chemocurable disease (Bast, 2011). Despite initially responding to treatment, most women diagnosed with HGSC develop recurrent disease and chemotherapy resistance (Pignata et al., 2017). The understanding of chemoresistance mechanisms has not yielded clinically usable alternatives, and EOC with platinum resistance is associated with poor overall survival (Ethier et al., 2017).

New treatment modalities such as targeted treatments are made possible by the exploration of underlying tumor biology. In 2000, researchers Hanahan and

Weinberg put forward a groundbreaking theory named hallmarks of cancer, in which the normal capacities and molecular mechanisms of a cell are capitalized upon and modified by cancer cells in order to acquire survival properties and other modalities to enhance spread and avoid normal protective mechanisms (Hanahan and Weinberg, 2000). These in turn can be exploited by means of targeted therapies (Hanahan and Weinberg, 2011). In the setting of ovarian cancer, understanding of the *BRCA* mutation and its effect on DNA repair, specifically the lack of a repair mechanism called homologous recombination (HR), has made possible the development of targeted inhibition of PARP.

PARP inhibitors have changed the therapy landscape for ovarian cancer, and most recent research demonstrates marked benefits in terms of primary treatment of *BRCA*-mutated ovarian cancer (Suh et al., 2018; Moore et al., 2018). However, more research is needed with respect to PARP inhibition and its possible predictive markers in order to identify all OC patients benefiting from PARP inhibitors (Mirza and Matulonis, 2017). The use of PARP inhibitors is now being expanded beyond tumors with HR deficiency to HR-competent tumors. These include tumors in which HR has been impaired synthetically by the use of other agents administered in combination with PARP inhibitors (del Rivero and Kohn, 2017).

The aim of this study was to explore the early pathogenesis of ovarian cancer and aspects of PARP inhibition and chemoresistance in clinical samples of HGSC patients and healthy *BRCA* mutation carriers (with no ovarian or breast malignancy).

2 REVIEW OF THE LITERATURE

2.1 Ovarian cancer

2.1.1 Epidemiology of ovarian cancer

Most ovarian cancers are of epithelial origin (90%), and only 10% are nonepithelial, originating from sex cord cells or from germinal cells (Webb and Jordan, 2017). Seventy percent of epithelial ovarian neoplasms are HGSC (Prat, 2012). The focus of this dissertation is primarily on HGSC.

Ovarian cancer is the deadliest gynecological malignancy among women worldwide, being the 7th leading cancer diagnosis and 8th leading cause of cancer mortality (Siegel et al., 2015). The incidence varies in different regions, with incidence being highest in Europe and North America and lowest in Thailand (Coburn et al., 2017). The prevalence of different subtypes, such as serous and endometrioid also varies, serous OC being more prevalent in Europe, and endometrioid OC in Asia. The incidence of OC is highest in Caucasian women, being highest in Northern Europe and the incidence rates have remained quite stable during the last 40 years (Coburn et al., 2017). In Finland, approximately 450 women are diagnosed yearly, but more than 250 women die of the disease each year (https://cancerregistry.fi).

The typical age at which diagnosis is received is 55 to 64, although the disease affects women in all age groups (Clarke-Pearson, 2009). The average lifetime risk of ovarian cancer is 1.37% in the general female population (Pearce et al., 2015).

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2.1.2 Risk and protective factors of ovarian cancer

Ovarian cancer has some generally accepted risk factors and in this chapter risk factors for OC are discussed, focusing also on the subtypes (especially HGSC) in some cases. The most prominent and well-known is the theory of lifetime number of ovulatory cycles associated with OC (i.e., incessant ovulation theory, in which the age at ovulation is an index of a woman's ovarian cancer risk) (Fathalla, 1971). Other suggested mechanisms include stimulation by hormonal exposures (including estrogens, insulin, androgens and IGF-1 as well as endometriosis, inflammation and hormone replacement therapy (HRT).

In more detail, it is well established that OC development involves pro-angiogenic factor-regulated angiogenesis, mainly mediated by vascular endothelial growth factor (VEGF) (Skirnisdottir et al., 2016). Notably, estradiol has a positive effect on VEGF expression in EOC, probably through the activation of the ERα receptor, which further accentuates the role of hormonal influence on the development of OC (Valladares et al., 2017). VEGF's role in OC development will be viewed in more detail in Chapter 2.3.3.1.

Chronic inflammation has also been suggested as a risk factor for OC (Ness and Cottreau, 1999; Risch and Howe, 1995), and a recent meta-analysis found that pelvic inflammatory disease (PID) might indeed be considered as a risk factor, although the analysis concluded that more prospective trials addressing the issue are needed (Zhou et al., 2017). In a study published in 2017, recurrent PID was found to carry two-fold risk for borderline ovarian tumors (Rasmussen et al., 2017). Interestingly, another study demonstrated higher risk of HGSC in association with PID (HR 1.47, 95% CI 1.04-2.07) (Stewart et al., 2018). The underlying hypothesis relates OC development to inflammation and repair (e.g., ovulation), hypothesizing that chronic inflammation might either directly influence the ovarian surface or, alternatively, influence the premalignant lesions in the Fallopian tubes (Kisielewski et al., 2013).

Endometriosis is another established OC risk factor, specifically of the endometrioid and clear cell subtype (Ruderman and Pavone, 2017). In a meta-analysis, the prevalence of ovarian cancer (endometrioid and clear cell type) among endometriosis patients was 2.0-17.0% (Heidemann et al., 2014).

In addition, both current and recent use of HRT in menopause are related to serous and endometrioid OC, but not mucinous or clear cell subtypes (Edmonds and Dewhurst, 2007). It is noteworthy, however, that HRT has been associated with a limited increase in overall cancer risk, and this increase in the risk of female reproductive organ cancers appears to be almost neutralized by a decreased risk of

gastrointestinal cancers; this finding has been especially evident in patients treated with estrogen alone (Simin et al., 2017).

While there are no strong associations of OC with tobacco use, there is a direct link for tobacco use in (borderline) mucinous cancers and a limited impact on overall OC mortality (Praestegaard et al., 2017). In addition, height and BMI are associated with the risk of OC, although the risk is specifically associated with non-HGSC, whereas prevalence of HGSC is unaffected by higher BMI (Dixon et al., 2016; La Vecchia, 2017). In a recent meta-analysis, it was concluded that high consumption of total, saturated and trans-fats increases OC risk and that different histological subtypes have different susceptibilities to dietary fat (Qiu et al., 2016). Increased alcohol consumption has been associated with OC risk (Yan-Hong et al., 2015; D. Wu et al., 2018); but interestingly, guideline-concordant consumption does not increase the risk, and it has been suggested that red wine consumption might even reduce the risk (Cook et al., 2016).

To summarize, there are some lifestyle associations with the risk of OC, but the strongest associations are found with ovarian functions, i.e., the menstrual cycle. The genetic risk factors are explored in detail in a different chapter (see 2.2.3). It is important to emphasize that OC, and especially HGSC, is not significantly affected by lifestyle choices unlike some other cancers, i.e. in case of lung cancer the association with smoking.

There are known protective factors that have long been associated with OC: oral contraceptives have been demonstrated to reduce ovarian cancer risk in several studies (Iversen et al., 2017; Wu et al., 2017). Additionally, the levonorgestrel-releasing intrauterine system (LNG-IUS) has been demonstrated to reduce the risk of both invasive (mainly serous, but also mucinous and endometrioid subtypes) and borderline ovarian tumors (Soini et al., 2016). Breastfeeding has also been found to be inversely associated with the risk of OC (Li et al., 2014). More specifically, long-term breastfeeding duration (more than 6 months) has demonstrated a stronger protective effect, as well as full-term pregnancy (Li et al., 2014). In that regard, full-term pregnancy has been demonstrated to be more strongly inversely associated with type I than type II tumors (type I: relative risk (RR) 0.47 [95% confidence interval (CI): 0.33-0.69]; type II, RR: 0.81 [0.61-1.06]) (Fortner et al., 2015). Type I and II tumors and their differences are discussed in more detail in Chapter 2.1.4 of this dissertation.

Importantly, there is widely recognized and well-established evidence demonstrated in multiple studies that sterilization prevents OC. Specifically, Hankinson et al. observed in 1993 that OC risk among women who had a tubal

ligation was 77% lower compared to that of women with no tubal procedure (Hankinson et al., 1993). Since then, multiple analyses with longer follow-up have supported these original findings (Rice et al., 2014; Birmann et al., 2016). In addition, it was observed in the studies referenced above (and others not mentioned here) that hysterectomy is a protective factor for OC.

Seventy percent of all ovarian neoplasms are HGSC (Prat, 2012). Current understanding is that the origin of HGSC is in the distal Fallopian tube (Karnezis et al., 2017). Interestingly, in a population-based cohort study, data on women with previous surgery on benign indications (sterilization, salpingectomy, hysterectomy, and bilateral salpingo-oophorectomy [BSO], hysterectomy; n = 251,465) were compared with data on an unexposed population (n = 5,449,119) between 1973 and 2009. The results indicated that among women with previous salpingectomy, there was a significantly lower risk for OC (HR = 0.65, 95% CI = 0.52 to 0.81) as well as among women with previous hysterectomy (HR = 0.79, 95% CI = 0.70 to 0.88), sterilization (HR = 0.72, 95% CI = 0.64 to 0.81), and hysterectomy with BSO (HR = 0.06, 95% CI = 0.03 to 0.12). A 50% decrease in OC risk was associated with BSO relative to the unilateral procedure (HR = 0.35, 95% CI = 0.17 to 0.73, and 0.71, 95% CI = 0.56 to 0.91, respectively) (Falconer et al., 2015).

To summarize, it has been well established that removal of the Fallopian tubes reduces OC risk (Falconer et al., 2015). These observations have strengthened the STIC theory (discussed in detail in chapter 2.4 of this dissertation) of the development of ovarian cancer and provide a means to prevent this deadly disease.

An overview of the risk and protective factors associated with OC is provided in Table 1.

Table 1. Risk and protective factors associated with ovarian cancer.

Risk factor	Risk, hazard or odds ratio
Lifetime number of ovulatory cycles	OR 2.22, 95% CI: 1.07, 4.62
Inflammation (HGSC)	HR 1.47, 95% CI: 1.04, 2.07
Endometriosis	OR 1.34, standardized IR of 1.43-8.95
Current and recent use of HRT in menopause	RR 1.43, 95% CI 1.31-1.56
Tobacco use	HR 1.17, 95% CI 1.08-1.28
Height (for women>or=175 cm)	OR 5.3; 95% CI 1.5-19.1
BMI	OR 1.29, 95% CI 1.03-1.61 per 5 units BMI
High consumption of trans-fats	OR 1.30, 95% CI 1.08-1.57
Increased alcohol consumption	RR 1.03; 95% CI, 0.96-1.10
Protective Factor	
Oral contraceptives	OR 0.58; 95% CI, 0.46 to 0.73
Breastfeeding	OR 0.66; 95% CI, 0.57-0.76
Full-term pregnancy	OR (95% CI) for Type I [0.09 (0.04-0.24) Type II [0.66 (0.43-1.02)
Sterilization	HR 0.72, 95% CI = 0.64 to 0.81
Unilateral salpingectomy	HR 0.71, 95% CI = 0.56 to 0.91
Bilateral salpingectomy	HR 0.35, 95% CI = 0.17 to 0.73
Hysterectomy	HR 0.79, 95% CI = 0.70 to 0.88

2.1.3 Symptoms of ovarian cancer

Ovarian cancer is often diagnosed at a late stage because the symptoms of this disease are nonspecific and vague. Women may present with symptoms such as abdominal bloating, discomfort and pressure symptoms (nausea, colicky abdominal pain) (Edmonds and Dewhurst, 2007). Pelvic pressure symptoms such as frequent urination, pressure in the pelvic region, constipation or diarrhea may be present. At the late stage, symptoms can include pelvic resistance, ascites, lymphadenopathy, and pleural effusion (Edmonds and Dewhurst, 2007). Malignant ascites is a specific feature of EOC and should lead to prompt examination, regardless of the presence or absence of accompanying adnexal mass, since it is strongly associated with poor prognosis (Huang et al., 2013).

2.1.4 Diagnostics of ovarian cancer

Basic investigation consists of pelvic examination, transvaginal ultrasound (TVU) and cancer antigen 125 (CA 125) (Skates et al., 2017). Adnexal solid or cystic masses with ascites prompt a suspicion for OC, especially when cysts have solid or papillary properties and are bilateral (Menon, 2004). If findings are suspicious for OC, the patient should be promptly referred to a gynecologic oncologist (Doubeni et al., 2016).

The tumor marker for OC, CA 125, was first discovered in the blood of patients with specific types of cancers or some benign conditions (Bast et al., 1998). However, due to its limited specificity and sensitivity, CA 125 alone isn't viewed as an ideal biomarker for OC (Bottoni and Scatena, 2015). Specifically, the sensitivity and positive predictive value have been found to be 64.29% and 53.57% for stage I-II cancer patients and 91.43% and 88.57% for stage III-IV cancer patients, respectively (Zheng et al., 2018). It has been well established that simultaneous testing with CA 125 and TVU produces high false-positive rates (RR, 1.01; 95% CI, 0.96-1.06), inducing unnecessary surgeries to identify one true-positive (Buys et al., 2011). Combined detection of CA 125 with HE4 improves the sensitivity and specificity of OC diagnosis and also has clinical significance that can later guide treatment planning in a better way compared to that with CA 125 alone (Zhao and Hu, 2016; Goff et al., 2017). Evidence also suggests that HE4 seems to better predict recurrence than CA 125 (Scaletta et al., 2017).

Based on patient history, clinical features and tumor markers, several risk-calculating models have been developed (Skates et al., 1995). In the 1990s, the risk of malignancy index (RMI) was developed and later modified. The RMI takes into account the patient's age, ultrasound score, menopausal status, a clinical impression score and serum CA 125 level (RMI 2 = U × M × serum CA 125) (Jacobs et al., 1990; Tingulstad et al., 1996). An RMI cut-off level of 200 had a sensitivity of 85% and specificity of 97% in discriminating pelvic masses in the initial study; since then, a similar sensitivity and specificity have been reported (Niemi et al., 2017; Yanaranop et al., 2017). Additional prediction algorithms have been developed in pursuit of even more precise preoperative investigation - ROMA is a risk of malignancy index that is also used in OC, and it incorporates HE4 value in addition to CA 125 (Wei et al., 2016). It is considered an additional tool in the diagnosis of OC.

In suspicion of OC, patient should be referred to a specialized center for evaluation, where more advanced preoperative diagnostics, CT, MRI, PET/CT, PET/MRI and ascites cytology are used according to and catering to a specific

clinical situation and possibilities (Javadi et al., 2016; Khiewvan et al., 2017). Cytology washings are especially important in the diagnostic phase, since malignant ascites is a prominent feature in OC. In detail, ascites arises as a plasma exudate, and its formation is a result of an imbalance between the efflux and influx of fluid from the peritoneal cavity (Nagy et al., 1993). Many factors are attributed to this, such as increased microvascular permeability, vascular endothelial growth factors and the blockade of lymphatic drainage (Milliken et al., 2002). In terms of malignant ascites formation, the most prominent factor is VEGF, which specifically modulates peritoneal permeability by downregulating adhesion proteins via tumor-derived VEGF (Bekes et al., 2016). It has thus been hypothesized that early dysregulation of vascular permeability leading to ascites may be associated with advanced OC with aggressive tumor biology, prompting a search for VEGF-based biomarkers (Liang et al., 2013). *Summa summarum*, ascites is distinctly associated with malignant ovarian and peritoneal diseases, and cytological samples from ascites can greatly contribute to preoperative evaluation.

The staging of ovarian cancer is surgical, and preoperatively, only efforts to predict the stage and thus possible prognosis are made. Definitive staging is surgical and is discussed in more detail in Chapter 2.2.1.

2.1.5 Screening of ovarian cancer

Currently, there are no clearly defined anatomical steps in early tumor progression that would allow for the screening of precancerous lesions, unlike cervical or colorectal cancer (Crum et al., 2013). However, the theory of OC arising from the tubal epithelium has led to attempts to screen with intrauterine probe samples and tubal lavage washings (Menon et al., 2014; van der Steen et al., 2017). These methods, however, are not currently in clinical use. In addition, there has been an effort to clarify the value of yearly screening in terms of reducing OC death rates. In a recent RCT where patients were annually screened multimodally (ultrasound, CA 125) versus no screening, the analysis demonstrated that death rates from OC were reduced by screening ($p=0.021$), with an overall average mortality reduction of 20% (Jacobs et al., 2016). The study highlighted that a rising level of CA 125 rather than an elevated concentration above cutoff level, is an important and sensitive marker for early cancer detection. This result is indeed very encouraging considering the high mortality rates of OC; however, as the researchers themselves conclude, further exploration of this subject is needed, including assessment of cost-effectiveness.

2.2 Histopathological classification of epithelial ovarian cancer

Ovarian neoplasms can be divided into three major groups: benign, borderline and malignant. These include stromal, germ cell and epithelial tumors depending on the cell of origin of the neoplasm. Stromal and germ cell tumors differ histologically from epithelial tumors and are relatively rare (10% collectively) (Boussios et al., 2017).

Borderline ovarian tumors (BOT) are epithelial neoplasms. They are characterized by upregulated cellular proliferation without destructive stromal invasion (Silverberg et al., 2004). Similar to invasive carcinomas, there are six histologic subtypes based on the epithelial cell type, including serous and mucinous, endometrioid, clear cell, seromucinous, and borderline Brenner tumor (Seidman et al., 2004). Histologically, BOT is characterized by hierarchically branching papillae and pseudopapillae with paucicellular, edematous, or hyalinized fibrous stroma, enlined with epithelial proliferations that are architecturally complex (Hauptmann et al., 2017). Borderline ovarian tumors have been associated with microinvasion, lymph node involvement and noninvasive and invasive peritoneal implants (Seidman and Kurman, 2000). They are not, however, a precancerous state of HGSC (although they are considered a precancerous state of low-grade tumors).

EOC is a heterogeneous disease with specific epidemiological, phenotypical and molecular subtypes, including high-grade serous carcinoma (HGSC), low-grade serous, endometrioid, mucinous and clear cell carcinoma (Prat, 2012). Serous carcinomas are 70% of all EOC, with HGSC accounting for 70% of all ovarian malignancies (Kaku et al., 2003). Endometrioid and clear cell subtypes are the next largest groups, and ovarian carcinosarcomas are also classified as epithelial cancers with sarcomatous differentiation (Dubeau, 2008).

Serous carcinomas are characterized by solid, papillary glandular and transitional patterns, and thus the histological diagnosis is straightforward, helped by the typical morphologic features of serous carcinoma such as glands that are slit-like rather than smooth/round, with prominent cellular budding and bizarre nuclei (Ramalingam, 2016). Endometrioid carcinomas are associated with squamous differentiation, endometriosis and adenofibromatous background (Malpica et al., 2004). Historically, immunohistochemistry was not used for diagnosis of either HGSC or low-grade serous carcinoma (LGSC) (Seidman et al., 2004). There are some overlapping characteristics, and on the other hand, some distinct immunophenotypic features exist as well (Ramalingam, 2016). Both express paired box gene 8 (PAX8), WT1,

estrogen receptor (ER) and progesterone receptor (PR) expression (Kaldawy et al., 2016).

Historically, ovarian cancer has been graded I-III, but currently, low- vs high-grade classification is used as it represents the aggressiveness of the disease more effectively (Crum et al., 2013). The incidence details of different subtypes of EOC are listed in Table 2.

Table 2. Epithelial ovarian cancer subtypes.

Subtype	%
High grade serous	70 %
Low grade serous	5 %
Mucinous	3 %
Endometrioid	10 %
Clear cell	10 %
Carcinosarcomas	2 %

Currently, immunoprofiling is used in addition to standard histopathological diagnostics described above, especially to differentiate between HGSC and LGSC. The current grading system (high and low grade) in combination with immunoprofiling provided by IHC methods for different markers such as p53, ER and PR, WT1, p16 and ARID1A have significantly enhanced diagnostic accuracy and interobserver accuracy (Köbel et al., 2014). Due to this significant change in diagnostics in 2014, results of older and newer studies are challenging to compare. The accuracy of OC subtype documentation has improved markedly, and up to 20% of subtype estimates may be falsely documented in studies undertaken before 2014. It is particularly important to note this when interpreting results from studies using older datasets.

2.3 Standard treatment of ovarian cancer

The golden standard of epithelial ovarian cancer treatment is primary debulking surgery (PDS) combined with 6 cycles of platinum-based combination chemotherapy (Tate et al., 2017). In stages IIIB-IV, bevacizumab is added at a dose of 7.5 - 15 mg/kg for suboptimally debulked patients according to national guidelines (Gadducci et al., 2019). With cytoreductive surgery and combination chemotherapy being used increasingly, 5-year survival has improved from 37% in 1974–1976 to 46% in 1999–2005 (Bast, 2011).

2.3.1 Surgery in the primary treatment setting

Surgical treatment is the most important cornerstone of treatment in all stages of OC, aimed to ensure optimal cytoreduction and confirm the diagnosis with appropriate staging according to FIGO (du Bois et al., 2009). OC staging has two purposes: to provide standard terminology allowing a comparison of patient outcomes between centers and assignment of patients and their tumors to prognostic groups requiring specific treatments (Prat, 2015). Currently, during surgery, the extent of the disease is described, and tumor tissue samples are analyzed in order to provide the details for adequate staging. Extent of disease is considered the most important predictor of recurrence and survival (Maxwell and Mutch, 2017). OC staging is currently based on 2014 FIGO recommendations as presented in Table 3 (Prat, 2015).

In the surgical management of OC in the primary setting, primary debulking surgery (PDS) is performed. In preoperative assessment, operability and the possibility of radical surgery are assessed by a multidisciplinary team including an experienced surgeon, pathologist and radiologist. The aim is to perform radical surgery and remove all visible disease (R0, i.e., no residual tumor). By definition, an R1 result is achieved with 0-10 mm of residual tumor, and the R2 result means 10 mm or more of residual tumor is present. In addition to removal of the cancerous tissue, pelvic and para-aortic lymphadenectomies (LNDs) are performed; details of LND are discussed below.

In apparent stage I-II, staging is performed. An important part of PDS and staging is LND. It is imperative to perform LND to ensure accurate staging and thus make decisions regarding adjuvant therapy (Mikami, 2014). LND's importance stems from the knowledge that even in a very early stage of OC development, lymphatic spread can already occur. Thus, in approximately 20% of cases, LND leads to upstaging in these early stages, as involvement of the paraaortic lymph nodes is present even without the involvement of the pelvic lymph nodes (Young et al., 1983; Burghardt et al., 1991; Shimizu, 2004). However, according to a study published in 2017, in low-grade disease, upstaging due to lymph node involvement alone occurred in only 2.4% of patients, raising questions about the value of lymphadenectomy in those cases (Minig et al., 2017). This, however, is only thought to be the case in low-grade and not in high-grade tumors.

Table 3. 2014 FIGO ovarian, Fallopian tube, and peritoneal cancer staging system and corresponding TNM. Copyright (2014), with permission from Elsevier (Prat, 2015).

FIGO Stage	Description	TNM
I	Tumor confined to ovaries or Fallopian tube(s)	T1
IA	Tumor limited to one ovary (capsule intact) or Fallopian tube, no tumor on ovarian or fallopian tube surface, no malignant cells in the ascites or peritoneal washings	T1a
IB	Tumor limited to both ovaries (capsules intact) or Fallopian tubes, no tumor on ovarian or fallopian tube surface, no malignant cells in the ascites or peritoneal washings	T1b
IC	Tumor limited to one or both ovaries or Fallopian tubes, with any of the following: IC1 Surgical spill intraoperatively, IC2 Capsule ruptured before surgery or tumor on ovarian or fallopian tube surface, IC3 Malignant cells present in the ascites or peritoneal washings	T1c
II	Tumor involves one or both ovaries or Fallopian tubes with pelvic extension (below pelvic brim) or peritoneal cancer (Tp)	T2
IIA	Extension and/or implants on the uterus and/or Fallopian tubes/and/or ovaries	T2a
IIB	Extension to other pelvic intraperitoneal tissues	T2b
III	Tumor involves one or both ovaries, or Fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes	T3
IIIA	Metastasis to the retroperitoneal lymph nodes with or without microscopic peritoneal involvement beyond the pelvis	T3a
IIIA1	Positive retroperitoneal lymph nodes only (cytologically or histologically proven)	T1,T2,T3aN1
IIIA1 (i)	Metastasis ≤ 10 mm in greatest dimension (note this is tumor dimension and not lymph node dimension)	T3a/T3aN1
IIIA1 (ii)	Metastasis > 10 mm in greatest dimension	
IIIA2	Microscopic extrapelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes	T3a/T3aN1
IIIB	Macroscopic peritoneal metastases beyond the pelvic brim ≤ 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes	T3b/T3bN1
IIIC	Macroscopic peritoneal metastases beyond the pelvic brim > 2 cm in greatest dimension, with or without metastases to the retroperitoneal nodes (Note 1)	T3c/T3cN1
IV	Distant metastasis excluding peritoneal metastases	Any T, Any N, M1
IV A	Pleural effusion with positive cytology	Any T, Any N, M1
IV B	Metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of abdominal cavity) (Note 2)	Any T, Any N, M1

In stages III-IV, PDS is also an aim. In stages IIIC-IV, assessment is made regarding whether the patient benefits from PDS or should undergo neoadjuvant treatment (NACT) in case the tumor is unresectable. For details of NACT, please see chapter 2.3.2.2 of this dissertation. It is important to convey that if R0 or R1 is not achievable, the patient does not benefit from surgery in terms of survival (du Bois et al., 2009). The maximum diameter of the postoperative residual tumor after cytoreductive surgery is considered to be the strongest independent clinical prognostic factor (Eisenkop et al., 1998). According to a meta-analysis published in 2002, a 5.5% increase in survival time (median) was associated with a 10% increase in maximal tumor resection. In detail, patients with ≤ 25% maximal tumor resection had a survival time (median) of 22.7 months, and patients with more than 75% maximal tumor resection had a survival time of 33.9 months. Thus, survival increased 50% depending on surgical outcome (Bristow et al., 2002). More recently, these results have been re-evaluated, and it is currently believed that surgical tumor reduction to 0 mm is the most important prognostic factor of survival (Chang et al., 2013; Tate et al., 2017; Cordeiro Vidal et al., 2017; du Bois et al., 2009).

To describe radical surgery, it includes a longitudinal abdominal incision extending from symphysis to sternum in order to allow a detailed exploration of the abdomen, in which special attention needs to be paid to the domes of the diaphragm, the entire small and large intestine and the peritoneum (Di Saia et al., 2018). At minimum, the following surgical procedures are performed: BSO, total extraperitoneal hysterectomy, cytology washings and multiple peritoneal biopsies, infragastric omentectomy as far as the splenic hilum, and systemic pelvic and para-aortic LND up to the level of the renal vessels bilaterally (Sehouli, 2014). Any metastatic structures are removed. In the HGSC setting, appendectomy is not necessary, as it is recommended for only mucinous tumors.

The relevance of LND in stage III disease has been of great interest, since it has been under dispute whether or not R0 debulked patients benefit from additional lymphadenectomies. This is an important matter, considering additional morbidity and mortality that might accompany more extensive surgery. In a well-designed study from 2006, removal of lymph nodes in patients with residual disease near 10 mm was evaluated, and according to the outcome, LND for patients with complete or near-complete resection of abdominal disease appears to be justified (Aletti et al., 2006). According to the prospective randomized LION study by the German Association Of Gynecological Oncology (AGO, Arbeitsgruppe der Gynäkologischer Onkologie), it appears that in patients with advanced OC for whom complete cytoreduction was achieved, additional systematic LND of clinically

negative lymph nodes did not provide additional benefit and should thus be omitted (Harter et al., 2019).

Ultraradical (extensive) cytoreductive surgery in ovarian cancer treatment has been proposed for patients with widespread disease (Ang et al., 2011). Specifically, patients with upper abdominal disease affecting the diaphragm, liver, spleen and omentum or widespread disease affecting the bowel will need much more radical surgery than the standard procedure described above in order to achieve complete or optimal cytoreduction (Ang et al., 2011). This ultraradical procedure often includes bowel resection, splenectomy, liver resection or mobilization and diaphragmatic stripping. The range of surgical techniques demanded to be able to perform these procedures is broad and use of the techniques is decided depending on the particular involvement of different organs. Mobilization of the liver in order to achieve peritonectomy in the upper abdomen is especially challenging (Sehouli, 2014).

In the setting of ultraradical OC surgery, supportive care demands are more extensive (Eisenkop and Spirtos, 2001). However, even though complete cytoreduction is the objective of PDS, tumor load remains an independent poor prognostic factor reflecting a more aggressive disease (Martinez et al., 2016).

In conclusion, it must be emphasized that surgical effort with a good outcome carried out skillfully is beneficial for the patient, as the 5-year survival rate of R0 disease regardless of the stage is up to 60% (May et al., 2017; Wimberger et al., 2007).

2.3.2 Adjuvant chemotherapy in first-line treatment

By definition, adjuvant therapy is additional treatment given after primary treatment in order to prevent recurrence and may include chemotherapy, radiation therapy, hormone therapy, targeted therapy, or biological therapy (Hayat, 1984). If there is disease is left behind postsurgery, further treatment is not adjuvant by definition.

The combination of paclitaxel and carboplatin is the standard combination of first-line chemotherapy for EOC stages IB-IV (Sehouli, 2014), and in stages IIIB-IV, according to national guidelines, bevacizumab is added (Gadducci et al., 2019). As introduced above, the decision to administer chemotherapy is made according to FIGO stage, which is crucial in contemplating the choice of options. Additionally, grading of the tumor is integrated in the assessment. Because there is no survival benefit, stage I A low-grade tumors do not require chemotherapy, but postsurgery

combination chemotherapy is recommended for high-grade stage 1A and for all other stages (IB-IV) (Trimbos et al., 2003).

Combination chemotherapy has improved the prognosis of ovarian cancer patients dramatically. The first-line treatment is platinum-based, a combination of platinum and taxane. The benefit of this approach was first established in the 1990s in the GOG 111 study, where patients with stage II-IV OC were randomized to receive paclitaxel-cisplatin vs cyclophosphamide-cisplatin, and in the paclitaxel-cisplatin arm, the response rate was 73% compared to 60% in the cisplatin-cyclophosphamide arm. PFS and OS were significantly longer with paclitaxel-cisplatin: 12.9 vs 17.9 months and 37.5 vs 24.4 months, respectively (McGuire et al., 1996). Following studies looking into combination chemotherapy, the superiority of one compound over others was investigated, as well as the role of topotecan, pegylated liposomal doxorubicin and gemcitabine combined with carboplatin. However, their efficacy did not differ from that of carboplatin and paclitaxel (Bookman et al., 2009). In comparison to platinum compounds, the superiority of carboplatin over cisplatin was demonstrated not in terms of treatment effect, because both are effective, but in terms of side effects, as carboplatin has fewer adverse effects (Greimel et al., 2006). In the SCOTROC trial, the effectiveness of docetaxel and carboplatin was demonstrated; the overall response rate for the study was 66%, and the median PFS was 16.6 months (95% CI 13.3–19.1), being comparable to the effectiveness of paclitaxel and carboplatin (Vasey et al., 2001). In addition, to further determine if there is any added benefit to adding a third chemocompound to the regimen, the OCTOPUS trial briefly mentioned above was conducted, and it demonstrated that compared to standard treatment, the addition of a third cytotoxic agent provided no benefit in PFS or OS, regardless of the surgical result (Bookman et al., 2009). Currently, the standard is 6 cycles of paclitaxel at 175 mg/m2 combined with carboplatin AUC (area under the curve) 5, providing the patient can tolerate this dosage (Marth et al., 2017). The recommended duration of the combination chemotherapy is six cycles.

Dose-dense therapy has also been tested with both agents. Compared with paclitaxel administered every 3 weeks, weekly paclitaxel did not prolong progression-free survival among patients with ovarian cancer (Chan et al., 2016). Dose-dense paclitaxel in combination with carboplatin was compared to a standard regimen in a large study including 600 patients, and this demonstrated a significant survival benefit: the median PFS was 28 months in the dose-dense arm vs 17.2 months in the standard regimen arm (Katsumata et al., 2009). Since it involves higher toxicity, and because the promising results of the Japanese study have not been repeated in

trials in Europe, the dose-dense treatment regimen is not currently considered a first-line option (Clamp AR et al., 2017).

Another investigated treatment option is intraperitoneal chemotherapy (IP). In 2006, GOG-172 was published, where paclitaxel-cisplatin was compared to iv paclitaxel and IP cisplatin on day 2 and IP paclitaxel on day 6 in the setting of advanced OC with an R0 or R1 surgical result. The treatment was administered in 3-week cycles, with 6 cycles in total. As a result, the 4-year survival was 65.6 months in the IP arm vs 49.7 months in the standard treatment arm (Armstrong et al., 2006). However, the adverse effects were also significant, and the quality of life of the patients in the investigative arm was poorer. Another study also demonstrated a survival benefit (28 months in the experimental arm vs 22 months in the standard treatment arm), but significant toxicity was also encountered, and IP chemotherapy is thus not currently part of the routine management of OC (Markman et al., 2001).

Hyperthermic intraoperative intraperitoneal chemotherapy (HIPEC) is a single treatment of intraoperative chemotherapy administered intraoperatively (Di Saia et al., 2018). This modality might add benefit in several ways: when the chemotherapy is given intraoperatively, drug exposure is better without the effect of postoperative adhesions; the surgeon can oversee optimal distribution of the drug; and hyperthermia increases DNA-crosslinking and tumor penetration, maximizing the effect of the drug administered (Cowan et al., 2017). Results of a prospective randomized controlled trial (RCT) were recently published (van Driel et al., 2018). In this study, 245 patients with stage III OC who were receiving neoadjuvant chemotherapy were randomized to HIPEC and standard treatment. The HR for the HIPEC group was 0.66, the 95% CI was 0.50-0.87 and the median recurrence-free survival was 10.7 months in the standard treatment group and 14.2 months in the HIPEC group. Other RCTs examining this approach, especially with regard to treatment toxicity, are awaited.

2.3.2.1 Bevacizumab in first-line treatment

Angiogenesis provides an important means for targeted therapy in EOC. Tumor nodules cannot increase to >1 mm without developing their own blood supply (Bast, 2011). OCs produce multiple angiogenesis-stimulating factors, such as VEGF, bFGF and IL-8 (Bast et al., 2009). Studies indicating that increased levels of VEGF in OC were associated with increased resistance to chemotherapy and a poorer prognosis have provided the molecular rationale for the use of antiangiogenic therapy (Siddiqui et al., 2011; Rojas et al., 2016).

In first-line treatment, the results from GOG-218 and ICON7 have demonstrated improved outcome when bevacizumab is administered in combination with standard chemotherapy and when continued after finalizing the initial treatment as maintenance. In GOG-218, 1873 patients with stage III-IV OC were recruited, and the primary endpoint was PFS. The main result demonstrated that the use of bevacizumab during and after carboplatin and paclitaxel chemotherapy (cycles 2-22) prolonged the median PFS by 3.8 months in patients with advanced epithelial ovarian cancer (10.3 months in the standard treatment arm and 14.1 months in the experimental bevacizumab arm) (Burger et al., 2011).

ICON 7 recruited 1520 patients with OC; 70% had stage IIIC or IV ovarian cancer. As a result, PFS at 42 months was 22.4 months without bevacizumab versus 24.1 months with bevacizumab, and significantly, in patients with high risk for progression, the bevacizumab benefit was a greater PFS at 42 months (18.1 months vs 14.5 months with standard therapy alone), with respective median overall survival of 36.6 and 28.8 months (Perren et al., 2011). The final report of ICON 7 showed the treatment benefit in terms of OS not in the overall analysis but only in poor-prognosis patients in stage IIIC and IV (Oza et al., 2015).

Thus, a Cochrane Systematic review stated that for patients with a high risk of progression, treatment with VEGF-inhibitors and chemotherapy improved PFS and OS (Wang et al., 2018). However, VEGF-inhibitor use also increased the incidence of common adverse events, and there was no survival benefit in the pure maintenance setting (Wang et al., 2018).

2.3.2.2 Neoadjuvant chemotherapy

NACT is used when successful PDS is considered unachievable, usually in stages IIIC or IV. It consists of 2-4 cycles of platinum-based chemotherapy prior to interval debulking surgery (IDS) (Wright et al., 2016). There is an ongoing debate about PDS vs NACT-IDS, and to date, the absolute superiority of either option has yet not been demonstrated (Angeles et al., 2018).

In a trial addressing the issue, 670 patients with stage IIIC-IV EOC were recruited. The HR for death (intention-to-treat analysis) in the NACT group followed by IDS, compared with the group assigned to PDS followed by standard chemotherapy, was 0.98 (90% CI, 0.84 to 1.13), and the HR for progressive disease was 1.01 (90% CI, 0.89 to 1.15). The complete resection of all macroscopic disease (at primary or interval surgery) was the strongest independent variable predicting OS (Vergote et al., 2010). In terms of complications, the patients in the IDS group fared

better: postoperative death (defined as death <28 days after surgery) occurred in 2.5% of patients in the PDS group and in 0.7% of patients in the IDS group. Grade 3 or 4 hemorrhage occurred in 7.4% of patients after PDS and in 4.1% after IDS, with infection in 8.1% and 1.7%, respectively. However, quality of life assessments did not differ at any point of the study.

The CHORUS trial was an RCT comparing NACT + IDS to PDS and standard chemotherapy. The results of this study were concordant with those of previously published RCT investigations. According to the CHORUS trial, the median OS was 22.6 months in the PDS group vs 24.1 months in the NACT group. However, in the PDS group, grade 3 or 4 postoperative adverse events and deaths were detected at a higher rate compared to that in the NACT group (24% vs 14%, respectively) (Kehoe et al., 2015).

A Cochrane Systematic review stated in 2012 that the use of NACT in women with stage IIIC/IV OC is an alternative to PDS, especially in the case of bulky disease (Morrison et al., 2012). PDS is however the standard in stage IIIA and IIIB (Morrison et al., 2012). According to a more recent Cochrane Systematic review, in terms of the survival rates of women with advanced OC, there is no conclusive evidence for IDS superiority. The benefit of IDS has only been demonstrated in case the surgery was not as extensive of primarily not performed by gynecological oncologist (Tangjitgamol et al., 2016).

Although the noninferiority of NACT has thus currently been accepted (Qin et al., 2018), more well-designed prospective RCTs and the reliable data they provide are needed. NACT and its effect on survival remain a target for active research. Currently, a trial investigating NACT and IDS vs PDS and standard chemotherapy (TRUST) is recruiting. There are many unanswered questions regarding the superiority or inferiority of NACT, and the possible induction of chemoresistance and difficulty of assessing the residual tumor during IDS remain the focus of the discussion, hopefully answered in the future by data provided from RCTs and basic research.

Targeted therapies, specifically bevacizumab, have been investigated in this setting as well: NACT+ bevacizumab following IDS has been proposed and may prove to be an acceptable strategy in the future, although further RCT data are still needed (Komiyama et al., 2018).

2.3.3 Treatment of recurrent ovarian cancer

As stated earlier, OC is a chemoresponsive, but almost never chemocurable disease (Bast, 2011). Most women diagnosed with HGSC develop recurrent disease and eventually also chemotherapy resistance, despite initially responding to treatment (Cornelison et al., 2017). Chemoresistance is defined by the timing of the recurrence: sensitive disease is defined by recurrence >12 months after completion of platinum-based first-line therapy and resistant disease by recurrence ≤6 months after completion of platinum-based first-line therapy (Di Saia et al., 2018). Partially sensitive is by definition a recurrence 6–12 months after completion of platinum-based first-line therapy (Mutch and Prat, 2014). The chemoresistance cycle is further characterized by recurrent relapses with shortening time intervals between relapses until the disease becomes untreatable, i.e., chemoresistant. Definitions to describe the events of treatment response have evolved over the course of time, and currently, a definition using the platinum-free interval (PFI) to categorize patients as having platinum-sensitive/resistant disease has been replaced by the therapy-free interval (TFI), which can be subgrouped into TFIp (PFI), TFInp (non-PFI) and TFIb (biological agent-free interval) (Wilson et al., 2017).

In platinum-sensitive recurrent disease, several chemotherapeutic agents have proven beneficial, and choosing the best option involves taking into account prior systemic therapy, *BRCA* status, tumor histology, the PFI and previous treatment with bevacizumab (Pignata et al., 2017). In platinum-sensitive disease, the CALYPSO trial has demonstrated that carboplatin and liposomal doxorubicin (CPLD) is associated with less toxicity and improved PFS compared with carboplatin and paclitaxel using standard doses (11.3 months in the experimental arm vs 9.4 months in the carboplatin and paclitaxel arm) (Pujade-Lauraine et al., 2010). Additionally, according to the OCEANS trial, bevacizumab with carboplatin and gemcitabine did not increase PFS when compared with standard treatment alone in patients with recurrent disease (12.4 vs 8.4 months, respectively), although no OS difference has been observed (Aghajanian et al., 2012). The GOG-213 trial assigned patients to a standard carboplatin and paclitaxel regimen vs carboplatin and paclitaxel plus bevacizumab. The study also had a surgical arm, the results of which are still awaited. The primary endpoint was OS. Median OS in the chemotherapy plus bevacizumab group was 42.2 vs 37.3 months in the chemotherapy group, the difference was non-significant statistically. Adverse effects (grade 3 or worse adverse event) were more prominent in the chemotherapy plus bevacizumab group (96% vs 86%) than in the chemotherapy group (Coleman et al., 2017).

The treatment of recurrent platinum-resistant/platinum-refractory EOC with sequential single-agent salvage chemotherapy is superior to multiagent chemotherapy because of its less significant toxicity since multiagent treatment has no survival benefit in this setting (Oronsky et al., 2017). The administration of paclitaxel either weekly or three -weekly was assessed in an RCT, in which 55 patients were enrolled. For weekly vs three-weekly paclitaxel, the objective response rates were 27% and 16%, the median PFS were 7 and 4.5 months, and the median OS l were 15.5 and 12.5 months, respectively (Osman et al., 2016). Importantly, the treatments significantly improved the quality of life of the patients.

To evaluate the effectiveness of combining bevacizumab with chemotherapy, the AURELIA trial was initiated as the first randomized phase III trial, and it was observed that adding bevacizumab to chemotherapy significantly improved PFS without any new safety concerns (Pujade-Lauraine et al., 2014).

To conclude, recurrent disease, especially platinum-resistant recurrent EOC, poses a significant treatment challenge with modest survival benefits when compared to primary treatment.

2.3.4 Mechanisms of resistance to chemotherapy

As a characteristic of EOC, most women become resistant to chemotherapy at some point in the disease. Cancer cells are able to actively expel the chemotherapeutic agent via transport proteins, resulting in treatment resistance. A form of chemotherapy resistance is multiple drug resistance (MDR), which is diagnosed when cancer cells become resistant to not only the primary chemotherapeutic agent (platinum in the case of OC) but also to the next treatment lines (Ozben, 2006). This effectively prevents further treatment and dramatically worsens the prognosis (Januchowski et al., 2013).

When considering the background of treatment response, resistance to treatment is divided into the categories of intrinsic or acquired resistance, although distinguishing these mechanisms can be difficult (Cornelison et al., 2017). In intrinsic resistance cancer cells have innate ability to persist through the first exposure to treatment (Abdullah and Chow, 2013). Specifically, cancer cells may have mechanisms in place prior to treatment (de novo) to avoid response, for instance through limited drug uptake, detoxification of drugs or enhancing the efflux of drugs (Gottesman, 2002). Some of the mechanisms involved include modified aldehyde dehydrogenase (ALDH) activity and B-cell lymphoma-2 (BCL2)-related

chemoresistance (Abdullah and Chow, 2013). Tumor microenvironment has recently been studied extensively and specifically in OC, and tumor cells ensure via properties of intrinsic mechanisms (cytokine and bioactive lipid expression) that cancer-associated, mesenchymal stem cells and tumor-associated macrophages regulate chemoresistance as well as tumor growth, angiogenesis and dissemination (Thibault et al., 2014).

Acquired resistance is the result of exposure to treatment and subsequent evolution of cancer cells to an unaffected and persistent state (Cornelison et al., 2017). Acquired chemoresistance is based on the idea that most cells are sensitive to the chemotherapeutic drug, but then develop resistance through spontaneous mutations at variable rates, as proposed by the Goldie-Coldman hypothesis (Dembo, 1984). Acquired chemoresistance is facilitated by different mechanisms including genetic and epigenetic alternations of crucial genes (David W Chan and YS Ngan, 2012). Most anti-cancer drugs impair DNA synthesis, damage DNA in the nucleus or break down the mitotic spindles in the cells (David W Chan and YS Ngan, 2012). As a result, DNA mutations, deletions, amplifications, translocations occur, and when changes happen to crucial genes, such as *TP53*, *RB1* and *KRAS*, cancer cells become aggressive and chemoresistant (Jardin et al., 2009; Sankala et al., 2011; Wang et al., 2010). Several other genes such as Krüppel-like factor 6 (*KLF6*), *AGGF1* and *MFAP4,* have been proposed to be associated with chemoresistance in OC and also as markers for treatment response (Zhao et al., 2019; Chen et al., 2018). MicroRNAs have also been found to regulate crucial gene expression in the development of drug resistance (L. Zhang et al., 2016). In cell line models, evidence of miRNA-21 regulating cisplatin resistance by negatively targeting the PTEN/PI3K/Akt signaling pathway in OC has emerged (Yu et al., 2017). Another study demonstrated that miR-34a has a suppressive effect on OC cells via directly binding and downregulating *HDAC1* expression (Lv et al., 2018).

The clonal origin and mutational adaptations associated with recurrent disease are however, poorly understood (Castellarin et al., 2013), partly because the genomic characteristics of HGSC samples collected at initial surgery have been extensively studied, but also because the molecular features of recurrent disease are less well-explored due to the challenges of sample collection posttreatment (Christie and Bowtell, 2017). The tumor suppressors *RB1, NF1, RAD51B* and *PTEN* are commonly inactivated in HGSC by gene breakage and that contributes to acquired chemotherapy resistance (Rojas et al., 2016). *CCNE1* amplification commonly has been found in primary resistant and refractory disease and indicates HR proficiency (Patch et al., 2015). However, as of yet, these

characteristics are not utilized in clinical decision-making. In terms of broader analysis, both known and novel pathways are commonly mutated, and it is thought that recurrent disease arises from multiple clones that are also present in the primary tumor with negligible accumulation of new mutations during standard treatment (Castellarin et al., 2013). In a recent study, however, it was demonstrated that after a single line of platinum, there was huge variability between primary and recurrent tumors, underlining the need for HGSC biopsies collected at relapse to tailor treatment options to the underlying genetic profile (Lambrechts et al., 2016). Interestingly, all primary platinum-sensitive HGSCs remained HR-deficient in this study, suggesting that although platinum treatment does change a tumor's properties, it likely remains responsive to PARPi treatment. In addition, it is noteworthy that even a single line of platinum therapy contributes to chromosomal instability, leading to acquired resistance (Lambrechts et al., 2016).

2.4 *BRCA 1 /2* mutations

The *BRCA1* gene was cloned in 1994, and it is located in human chromosome 17q21 (Miki et al., 1994). The BRCA1 is a multifunctional protein and is known to interact with different protein partners in various cellular compartments and it plays essential roles in diverse cellular pathways such as DNA damage repair, apoptosis, cell cycle arrest, transcriptional activation, genetic instability and tumorigenesis (Deng and Scott, 2000). *BRCA2* is a large gene comprising 10,254 nucleotides and 26 coding exons (Perets et al., 2013) and is located in 13q13.1, which is the long (q) arm of chromosome 13 at position 13.1. BRCA2 suppresses genome instability by being centrally involved in two processes: DNA lesion repair by HR and nascent strand protection from degradation at stalled replication forks (Prakash et al., 2015). In different models, BRCA2 has been evaluated and it has been demonstrated that it prevents chromosomal aberrations upon replication stalling to maintain genomic integrity, and through this likely suppresses tumorigenesis (Schlacher et al., 2011).

BRCA genes have an essential role in suppressing genome instability: they behave as caretakers and thus act as chromosome structure preserves (Venkitaraman, 2002). The best-known function of both BRCA1 and BRCA2 is in the DNA repair pathway. In DNA, different lesions are constantly caused by UV light and metabolic processes as well as other factors. Among these different types of lesions, double-strand-breaks (DSBs) are repaired by several mechanisms but preferably by HR repair, which is the cell's most error-free repair mechanism. (Moynahan et al., 1999;

Bouwman and Jonkers, 2012; Severson et al., 2015). Cells without functional BRCA1 and BRCA2 are deficient in HR and are forced to use alternative mechanisms to repair DSBs. These highly error-prone non-homologous-end-joining (NHEJ) mechanisms, which result in genomic instability, further worsen the cell's state and ability to repair lesions (Severson et al., 2015). Ultimately, this results in genomic instability that is so significant that the cell is predisposed to cancer formation.

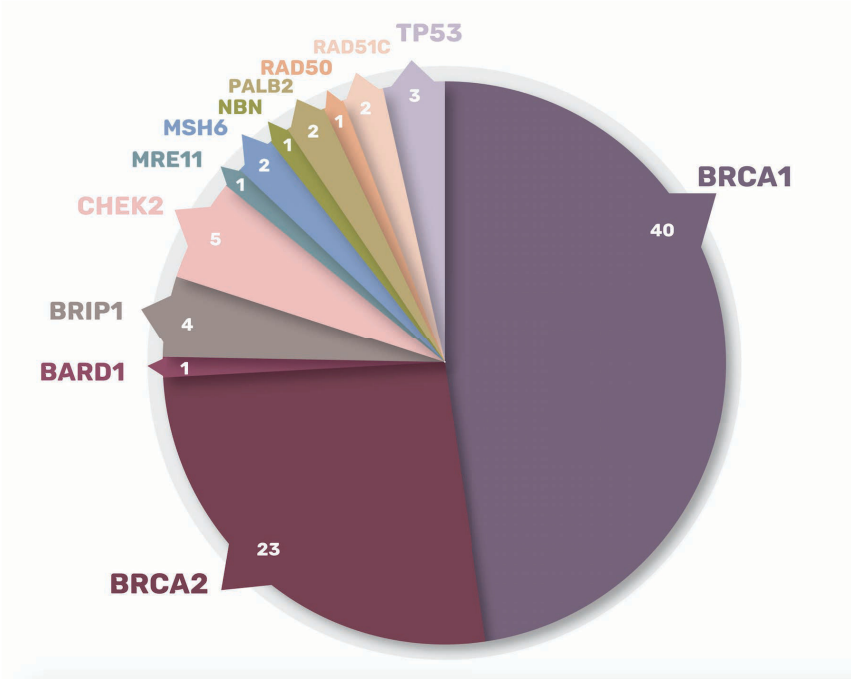
2.4.1 Hereditary ovarian cancer

In terms of hereditary breast and ovarian cancer syndrome, in 20% of HGSC cases, the disease is due to hereditary or somatic mutations in *BRCA1* and *BRCA2* (Edmonds and Dewhurst, 2007). The lifetime risk of breast cancer in *BRCA 1/2* mutation carriers is 41% to 90% (Kuchenbaecker et al., 2017; Ford et al., 1998). Approximately 65-85% of ovarian tumors with hereditary susceptibility (20% of all cases) have a *BRCA* gene germline mutation; nevertheless, some other suppressor genes and oncogenes have also been associated with hereditary OCs (Toss et al., 2015). These include mismatch repair (MMR) gene mutations in Lynch syndrome that is associated with clear cell and endometrioid subtypes (and not associated with *BRCA* mutations), the tumor suppressor gene *TP53* in Li-Fraumeni syndrome, and several other genes involved in the DSB repair system, such as *CHEK2, RAD51, BRIP1*, and *PALB2* (Nakonechny and Gilks, 2016; Walsh et al., 2011). In addition, *BRCA 1/2* mutations predispose to several other cancers, most prominently to pancreatic (Matsubayashi et al., 2017) and prostate cancers (Ibrahim et al., 2018). An overview of the genes involved is depicted in Figure 1.

In ovarian cancer, 40% of BRCA1 mutation carriers are diagnosed with the disease by age 70, and 18% of BRCA2 mutation carriers face risk (Kuchenbaecker et al., 2017; Venkitaraman, 2002). Ovarian screening does not improve outcomes, so BRCA mutation carriers are recommended to undergo prophylactic risk-reducing salpingo-oophorectomy (RRSO) around the age of 40, followed by hormone replacement therapy in the case of negative breast cancer history (Hartmann and Lindor, 2016). Any specimens from prophylactic surgery should be carefully examined for occult malignancy, which do occur and have provided additional confidence in the current counseling for RRSO for high risk women (Lee et al., 2017). The genetic testing in order to identify patients with inherited predisposition to cancer should be performed only after careful counseling, especially in case the testing panels for genes include those that may identify confusing variants of

uncertain significance or unsuspected disease predisposition (Society of Gynecologic Oncology, 2017). New methods for triaging patients for genetic testing are needed, as the selection criteria that is currently used, fails to detect up to half of mutation carriers (Nilsson et al., 2018).

Figure 1. Inherited ovarian cancer, proportions of genes with primary ovarian, Fallopian tube or peritoneal cancers with germline mutations. Adapted from PMC (Walsh et al., 2011)



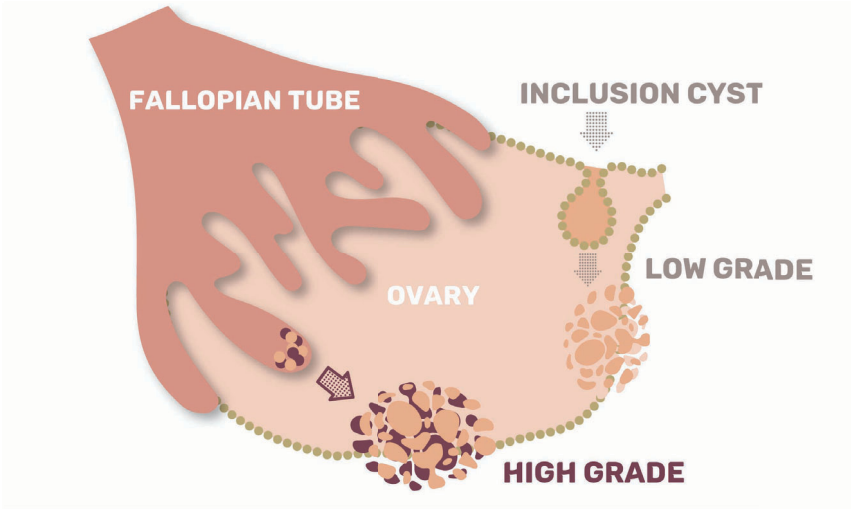
Currently, the most effective method for preventing OC is without a doubt primary surgical prevention through RRSO (Tschernichovsky and Goodman, 2017), especially among the high-risk patient population, such as *BRCA1* and *BRCA2* mutation carriers (Temkin et al., 2017). Thus, as a guideline, currently RRSO is recommended for both *BRCA1* and *BRCA2* carriers between the ages of 35 and 40 years who have completed their childbearing (Hartmann and Lindor, 2016; Edmonds and Dewhurst, 2007; Walker et al., 2015). In *BRCA2* mutation carriers, surgery might also be considered at a later age (up to 45 years), since the onset of cancer is 8 to 10 years later than that of *BRCA1* carriers (Marchetti et al., 2014), and the risk of developing OC before the age of 50 years is 1% (Hartmann and Lindor, 2016). Not insignificantly, the cost-effectiveness of RRSO in *BRCA1*/*BRCA2* carriers older than 35 years is also well established (Walker et al., 2015). In other

moderate-risk gene mutation carriers and those with polygenic risk, RRSO needs be considered at 50 years of age (Yamauchi and Takei, 2018). The optimal threshold for RRSO remains the subject of ongoing research and discussion, aimed to ultimately enable introduction of a targeted primary prevention approach that would significantly impact the future burden of OC (Manchanda and Menon, 2017). Further details of RRSO and BRCAness are detailed in Chapters 2.5 and 2.6.

2.5 RRSO and the origin of HGSC

It has been historically thought that the polarized cuboidal epithelial monolayer that is in continuity with the rest of the peritoneal mesothelium and covers the ovaries is the origin of EOC (Edmonds and Dewhurst, 2007). This theory has been challenged, and it is the present understanding that in fact OC arises from the tubal epithelium (Dubeau, 2008; Nishida et al., 2016; Piek et al., 2001) in the case of HGSC (Kurman and Shih, 2010). More distinctly, high-grade serous ovarian cancer refers to the HGSC arising from the ovary, Fallopian tube, or peritoneum (Kim et al., 2018).

Figure 2. Development of high grade epithelial ovarian cancer arising from fimbriae. Adapted from Gloss and Samimi (Gloss and Samimi, 2014).



Extensive and groundbreaking work exploring the origin of HGSC has demonstrated that STIC lesions are suspected to be the precursor for most HGSCs of the pelvis (Prat, 2012). This is based on an observation that there is a clonal relationship between STIC and established HGSCs sharing *TP53* mutations as well as integrated molecular analyses (Lee et al., 2007; Eckert et al., 2016; Ducie et al., 2017). The data have been retrieved from material derived from *BRCA* mutation carriers undergoing RRSO. In these, from 57% up to 100% of prophylactic surgeries in both the *BRCA1* and *BRCA2* mutation carrier populations reveal the involvement of the Fallopian tube, especially at the fimbriated end (Callahan et al., 2007; Finch et al., 2006).

Thus, STIC is an intraepithelial malignancy with potential for spread, and it directly precedes invasive serous carcinoma. It is composed of secretory cells having significant atypia, such as loss of nuclear polarity, increased nuclear to cytoplasmic ratio and prominent nucleoli (Crum, 2009). In another work, intermediate lesions– or "tubal intraepithelial lesions in transition" (TILTs) – in which p53-aberrant epithelium displays a higher degree of proliferative activity however falling short of malignancy, have also been described (Jarboe et al., 2008). The association studies referred to above have been followed by genetic model studies demonstrating that early alterations in *BRCA*, p53, and *PTEN* indeed lead to intraepithelial precursor lesions (Perets et al., 2013).

According to current understanding, implants are shed on the peritoneal surface epithelium from these lesions that arise from the distant Fallopian tube, giving rise to HGSC. Depending on where the tumorous cells start to proliferate, the disease is classified as high-grade serous ovarian cancer (HGSOC), high-grade tubal carcinoma or high-grade peritoneal carcinoma. Because of their origin and properties, they are essentially one disease with a similar course and prognosis (Kim et al., 2018). Several works have been published that support this theory, reviewed by Singh and colleagues (Singh et al., 2017).

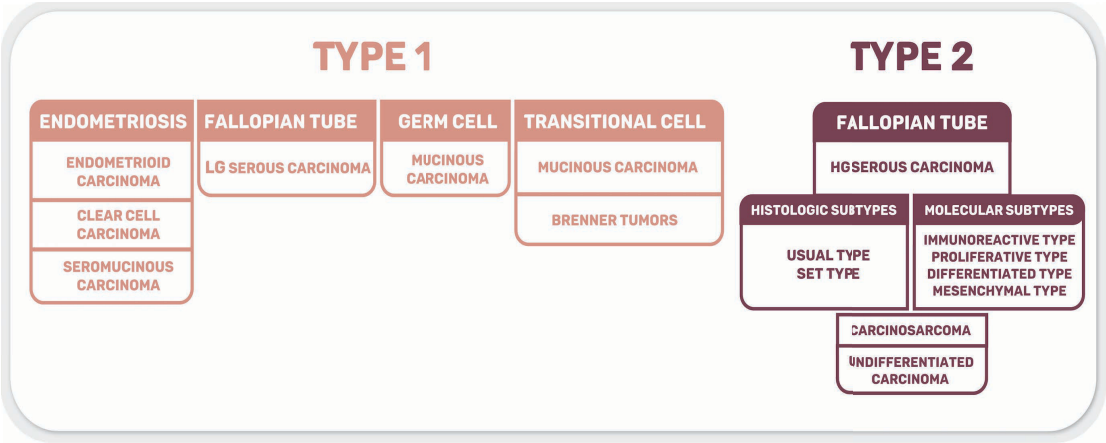
2.5.1 Characterization of low grade and high grade tumors

In EOC, tumors are divided in two subtypes: type I tumors comprise low-grade serous, low-grade endometrioid, clear cell and mucinous carcinomas, and Brenner tumors and are characterized by specific mutations, including *KRAS, BRAF, ERBB2, CTNNB1, PTEN, PIK3CA, ARID1A*, and *PPP2R1A* (Shih and Kurman, 2004; Kurman and Shih, 2011; Wiegand et al., 2010). Type II tumors include high-

grade serous, high-grade endometrioid (not illustrated in the figure), malignant mixed mesodermal tumors (carcinosarcomas), and undifferentiated carcinomas, which are characterized by aggressiveness, presentation in advanced stage, and a very high frequency of *TP53* mutations (Shih and Kurman, 2004). Type II tumors rarely harbor the mutations detected in type I tumors (Kurman and Shih, 2011). This subtype division is illustrated in Figure 3.

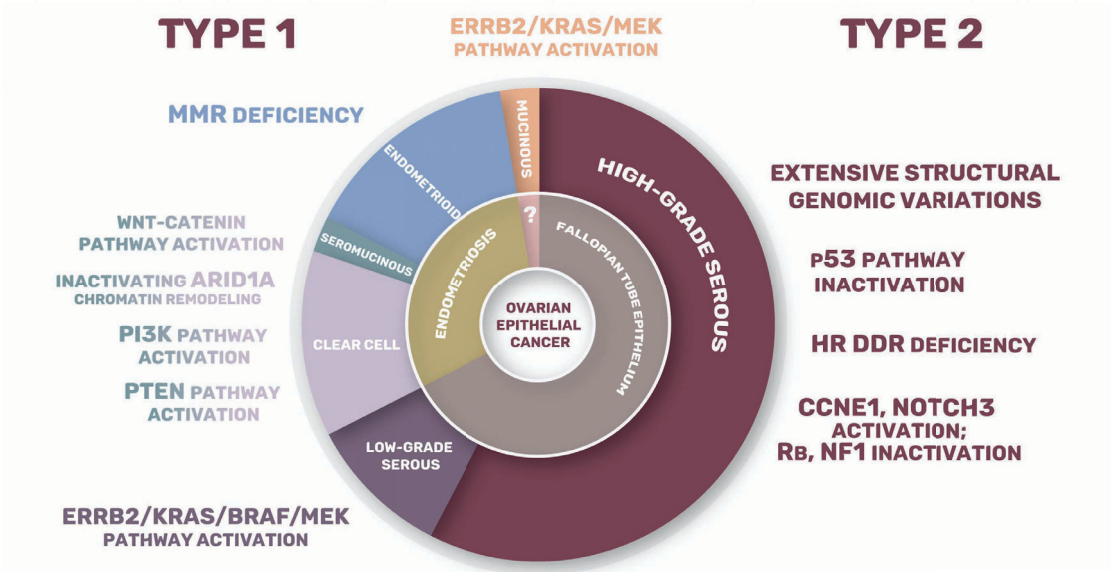
Figure 3. Expanded dualistic model of ovarian carcinogenesis. Ovarian carcinomas derive from endometrial tissue, Fallopian tube tissue, germ cells, and transitional epithelium. Type I carcinomas comprise endometrioid, clear cell, LG serous, and mucinous carcinomas. Type II carcinomas are largely composed of HG serous carcinoma, carcinosarcoma, and undifferentiated carcinoma. Transitional cell indicates metaplastic transitional epithelium at the tuboperitoneal junction. Adapted from Kurman and Shih (Kurman and Shih, 2016).

LG-low grade
HG – High grade
SET – solid, pseudoendometroid, transitional carcinoma



Furthermore, type II tumors have molecular alterations that perturb the expression of *BRCA* either by mutation or by promoter methylation and the defining characteristic of these tumors is genetic instability (Kurman and Shih, 2011). New molecular genetic data, especially those data derived from next-generation sequencing (NGS), further underline the heterogeneity of OC and identify actionable mutations in terms of possible targeted treatments (Binder et al., 2015).

Figure 4. The revised dualistic model in the pathogenesis of EOC. Type I carcinomas comprise low-grade serous, clear cell, endometrioid, and mucinous carcinomas. Seromucinous carcinomas and malignant Brenner tumors are rare and not shown. Type II carcinomas are largely composed of high-grade serous carcinomas. Carcinosarcoma and undifferentiated carcinoma are relatively uncommon and not illustrated. The molecular pathway alterations that characterize each tumor subtype are shown color-coded beside the subtype. Some pathway abnormalities are shared by different tumor types and are shown in two-color coding. Adapted from Kurman and Shih (Kurman and Shih, 2016).



ARID1A, AT-rich interaction domain 1A
BRAF, B-Raf proto-oncogene, serine/threonine kinase
CCNE1, cyclin E1
ERRB2, estrogen-related receptor β2
HR DDR, homologous recombination-mediated DNA damage repair
KRAS, Kirsten rat sarcoma viral oncogene homolog
MEK, mitogen-activated protein (MAP) extracellular signal-related kinase (ERK) kinase
MMR, DNA mismatch repair
NF1, nuclear factor 1
PI3K, phosphatidylinositol 3-kinase
PTEN, phosphatase and tensin homolog
Rb, retinoblastoma protein

An important feature of HGSC is that it has the tendency to spread from primary tumor cells to nearby organs such as bowel, peritoneum, uterus, mesentery and omentum (Weidle et al., 2016). HGSC also spreads through blood and lymph vessels.

To conclude, HGSC is currently viewed as an entity of three diseases – high-grade epithelial Fallopian tube cancer, high-grade epithelial ovarian cancer and primary peritoneal cancer (Singh et al., 2017). Molecular subtyping and constantly widening understanding of the underlying mechanisms of this disease entity have led to improvements in treatment strategies.

2.6 Molecular subtyping of EOC

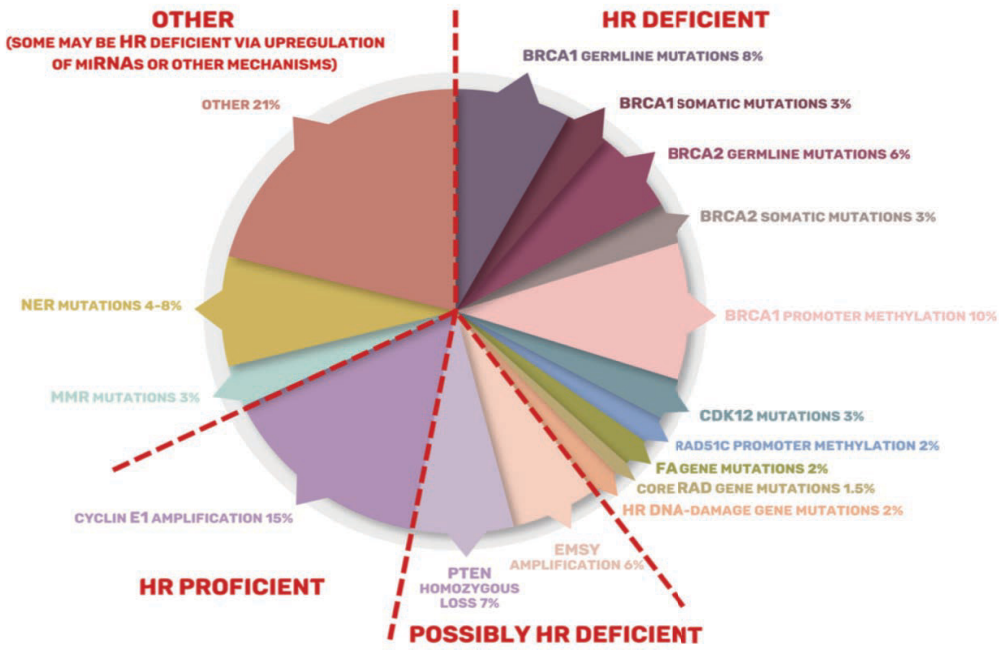
Although division of EOC into either type I or type II is currently based on histology and tumor grade, the development of molecular analyses will possibly provide a more accurate basis for tumor classification (Rojas et al., 2016). Using microarray technique, the gene expression profiling has separated four molecular subtypes of HGSC, each correlating with survival data and has specific biological characteristics: C1 (mesenchymal), C2 (immunoreactive), C4 (differentiated), and C5 (proliferative) (Patch et al., 2015; Leong et al., 2015).

Clustering analysis and extensive search in the existing literature have thus outlined four gene clusters that correspond to distinct biological processes, including cell division, tumor invasion, mitochondrial system and glycoprotein synthesis, the last of which is speculated to be highly predictive of OS among OC patients (H. Zhang et al., 2016). Tumor expression profiling with techniques such as sequencing, microarray, and proteomic methods have provided analysis at the DNA, RNA and protein levels, however, the challenge of determination of clinically significant alterations is significant (Binder et al., 2015). Currently molecular subtyping is not used in routine clinical praxis, due to its suboptimal repeatability.

The coding genes from 316 HGSCs have been sequenced by The Cancer Genome Atlas (TCGA) Research Network and 96% of the genes had somatic mutations of *p53, BRCA1* or *BRCA2* mutations (germline or somatic) were found in 20% (H. Zhang et al., 2016). *RB1, NF1, FAT3, CSMD3, GABRA6* and *CDK12* were also recurrently mutated in ≤5% of HGSCs (Zhang et al., 2014). Overall, ~50% of HGSCs exhibit HR defects, including tumors with epigenetic silencing, the *BRCA1* and *BRCA2* inactivating mutations, *EMSY* amplification or mutation, *PTEN* deletion or mutation, *RAD51C* hypermethylation, *ATM* or *ATR* mutation, and Fanconi anemia genes mutation (Bast, 2011). These genomic scar signatures are an important feature to consider, since they pose the possibility for targeted treatment as well as for response-related issues in platinum treatment (Konstantinopoulos et al., 2015). A genomic scar is defined as a genomic aberration

with a known origin, and in the setting of OC and HR, a genomic scar includes telomeric allelic imbalance (TAI) and loss of heterozygosity (LOH), which are thought to be biomarkers for homologous recombination deficiency (HRD) (Watkins et al., 2014). In 2018, Macintyre et al. explored copy-number signatures and found a relation to treatment response (Macintyre et al., 2018). HR deficiency and mutational signatures have also been investigated in terms of treatment response to PARP inhibitors beyond *BRCA* mutations and according to a recently published study, may be useful in PARP inhibitor- related decision-making, since HR deficiency signature is associated with good response to PARP inhibitors (Gulhan et al., 2019). The details of HRD are further discussed in Chapter 2.7 and 2.8. The data from TCGA are visualized in Figure 5.

Figure 5. Molecular subtyping of EOC: associated genes. Approximately 50% of HGSCs have alterations in HR repair genes. HR-deficient tumors on the right are associated with FA/BRCA pathway alterations. PTEN deletion and EMSY amplification are possibly HR-deficient. HR-proficient tumors (cyclin E1) are associated with inferior outcome and poor response to platinum-based chemotherapy. Remaining tumors may be HR deficient via miRNA upregulation or other unknown mechanism. Adapted from (Konstantinopoulos et al., 2015).

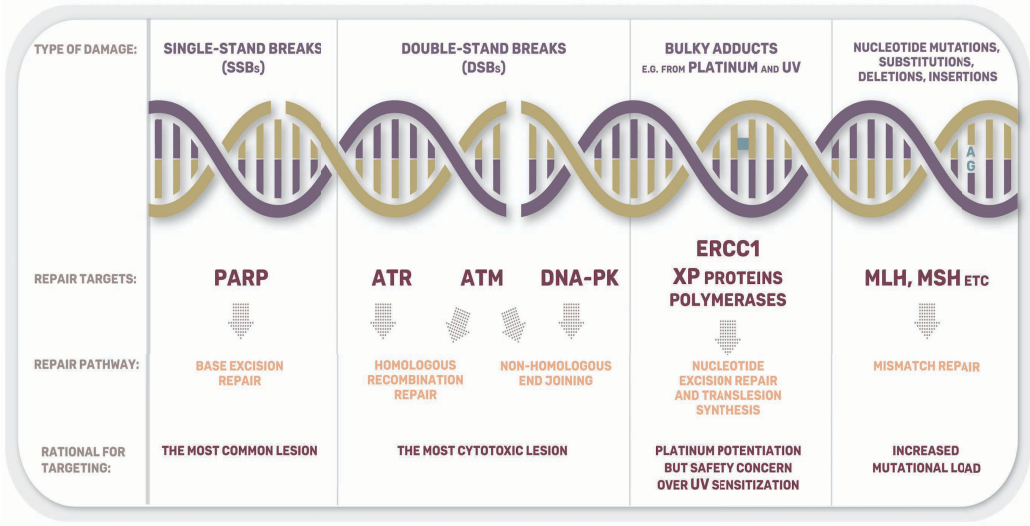


2.7 DNA damage repair and BRCAness

Lesions in genomic DNA are induced by both environmental and internal factors (Ciccia and Elledge, 2010), and failure to repair them will induce genomic instability and ultimately tumorigenesis (Li and Yu, 2015). Different DNA lesions include DNA strand breaks (double or single), chemically modified nucleobases, and intrastrand DNA cross-links (Geacintov and Broyde, 2017). Genomic stability is ensured by the DNA damage response system that recognizes and repairs DNA lesions, thus suppressing tumorigenesis (Jackson and Bartek, 2009; Li and Yu, 2015).

In more detail, alterations in the DNA can be divided into crosslinks, base modifications, single-strand breaks (SSBs) and double strand breaks (DSBs) (Caruso et al., 2017). DNA can be repaired by at least five different mechanisms, two of which are responsible for DSB repair: homologous recombination (HR) and nonhomologous end joining (NHEJ) (Davar et al., 2012). By ensuring efficient and precise repair of DSB, the BRCA1 and BRCA2 proteins are central to the role of maintaining genomic stability (Ledermann et al., 2016). Cells with intact BRCA are able to survive by repairing DSBs, but *BRCA*-mutated cells undergo apoptosis because of the inability to repair damage (Morris and Chan, 2015). By definition, alteration in two genes (either by a mutation or a medical compound) initiates a synthetically lethal interaction, essentially because the perturbation of either gene alone is survivable, but the perturbation of both genes simultaneously results in the loss of viability (Bhattacharjee and Nandi, 2017). The synthetic lethality and the underlying genetic interactions have been identified and mechanistically characterized, which greatly enhances the possibilities of exploiting synthetic lethality in cancer treatment (O'Neil et al., 2017).

Figure 6. DNA damage response pathways – drug targets. Cell cycle targets are shown with rationale for targeting these pathways. PARP, ATR, ATM and DNA-PK are SSB and DSB repair targets that are being evaluated in clinical trials. Adapted from O'Connor (O'Connor, 2015).



There is considerable progress made in the understanding of *BRCA1* and *BRCA2* gene functions and the loss-of-function mutations, making cells vulnerable to targeted therapy (Lord and Ashworth, 2016). "BRCAness" is the functional state and therapeutic sensitivity that is often referred to in this state, and it is most commonly associated with OC, but also some breast cancer types (Li et al., 2017). The 'BRCAness' properties are essentially the characteristics that some sporadic malignancies share with those occurring in either *BRCA1*- or *BRCA2*-mutation carriers, such as response to standard therapy (Turner et al., 2004). Prior investigations have shown that a BRCA-like phenotype can be present in approximately 50% of cases of HGSC, most likely reflecting HRD status (Tan et al., 2008; M. Zhang et al., 2016).

2.8 PARP inhibition

The first clinically approved drugs developed to exploit synthetic lethality are the PARP inhibitors (PARPi), a cancer therapy targeting poly(ADP-ribose) polymerase (Lord and Ashworth, 2017). Poly(ADP-ribosyl)ation (PARylation) is an integral part of the DNA damage response system and it has multiple roles in several molecular and cellular processes (Taylor and Eskander, 2018). In addition to DNA damage detection and repair, it also has a role in mitotic apparatus function, chromatin modification, cell death pathways, transcription, and insulator function (Schiewer et al., 2012). Genome maintenance, aging, carcinogenesis, inflammation, and neuronal function are some of the physiological and pathological outcomes that critically depend on these processes (Kim et al., 2005).

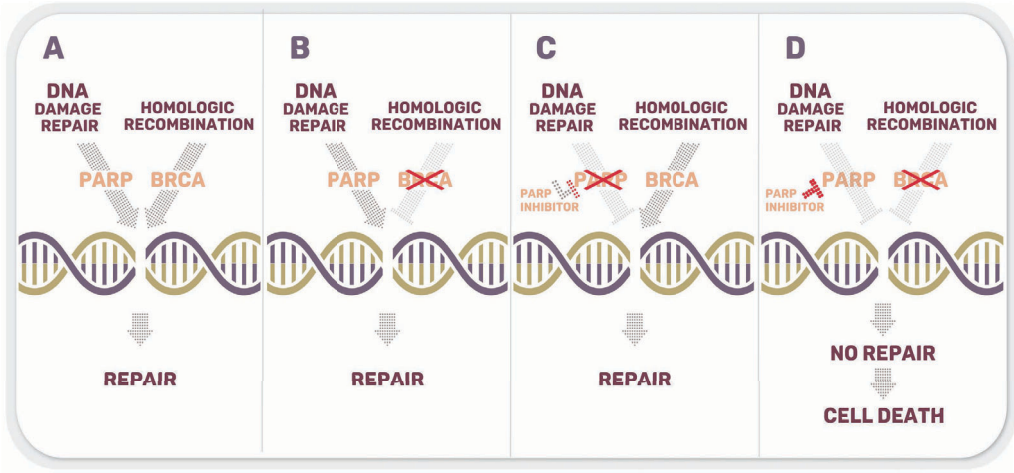
In terms of the DNA damage response and OC, PARylation regulates both DSB and SSB repair and cell cycle checkpoint activation (Schiewer et al., 2018). DNA damage-induced PAR is recognized by many DNA damage response factors. PAR mediates the recruitment of DNA damage response factors to the lesions and this initiates DNA damage response pathway activation (Pascal, 2018). After activation of different DNA damage response pathways, the PAR is degraded by PARG and other mechanisms (DNA damage-induced phosphorylation and ubiquitination events) ensure that DNA repair factors are selectively retained at DNA lesions to complete repair (Li and Yu, 2015). Cells with BRCA1 or BRCA2 dysfunction are profoundly sensitized to the inhibition of PARP enzymatic activity that leads to chromosomal instability, cell cycle arrest and eventually, apoptosis (Farmer et al., 2005). DSBs repair is mediated by two main pathways, NHEJ and HR, and three main pathways are used to repair SSBs: nucleotide excision repair, base excision repair (BER), and mismatch repair (Hengel et al., 2017). PARP1 is involved in several pathways such as NHEJ, HR, and BER (Li and Yu, 2015).

As described above, BRCA1 or BRCA2 loss results in defective HR repair of DCBs and repair therefore relies on potentially mutagenic mechanisms such as NHEJ (Taylor and Eskander, 2018). These explorations have introduced the synthetic lethality approach to targeting *BRCA*-mutated cancers, with PARP inhibition as a well-researched example as described above (Lord and Ashworth, 2013). To further illustrate, a normal cell survives PARP inhibition because it is able to use other repair mechanisms for the damage that has occurred. However, in BRCA-mutated cells (or otherwise HR deficient), the ability to repair SSBs via BER is lost if PARP1 is inhibited, resulting in cell death (synthetic lethality). An illustration

of DNA damage and its repair in normal cells and BRCA-mutated cells is provided in Figure 7.

Figure 7. DNA damage repair in normal cell and HRD cell demonstrating the PARP inhibition mechanism in normal and BRCA-mutated cell. Adapted from Mäenpää (Mäenpää, J, 2018).

A - normal cell, B- BRCA-mutated cell, C – PARP inhibition in normal cell, D – PARP Inhibition in BRCA-mutated cell



PARP inhibitors have been a much-awaited breakthrough in the treatment of OC. The first PARPi approved for cancer treatment was olaparib in 2014 (Dréan et al., 2016). Currently, olaparib, rucaparib and niraparib have also been approved in the US and/or Europe for the treatment of *BRCA*-mutated OC. There are many ongoing RCTs exploring different combinations of PARPi with chemotherapeutics or molecular-targeted agents (Ohmoto and Yachida, 2017). Interestingly, it has also been hypothesized that in patients with *BRCA1/2* mutation carrier OC (PBMCOC), exposure to PARPi may compromise benefit to subsequent chemotherapy with platinum-based regimens, possibly through the acquisition of secondary *BRCA1/2* mutations (Barber et al., 2013). However, Ang et al. found in 2013 that heavily pretreated PARPi-resistant PBMCOC retains the potential to respond to subsequent chemotherapy, and no secondary *BRCA1/2* mutations were found (Ang et al., 2013).

Excitingly, recently PARP inhibition has shown its importance in first-line treatment according to the SOLO1 trial, in which women with newly diagnosed advanced OC and a *BRCA1/2* mutation showed substantial benefit in PFS with olaparib maintenance in frontline therapy. In detail, after a median follow-up of 41 months, olaparib lowered the risk of disease progression or death by 70% compared to placebo (Kaplan–Meier estimate of the rate of freedom from disease progression and from death at 3 years, 60% vs 27%; HR for disease progression or death, 0.30; CI 95% 0.23 to 0.41; P<0.001) (Moore et al., 2018).

In the treatment of recurrent disease, the much anticipated results from three phase III trials (NOVA, SOLO2 and ARIEL3) demonstrated remarkable survival benefit (improvement in PFS) when PARP inhibitors were given as maintenance therapy to patients with complete or partial response after platinum-based therapy for platinum-sensitive OC (Mirza et al., 2018; Pujade-Lauraine et al., 2017; Coleman et al., 2017). An overview of these trials is detailed in Table 4.

The results of first-line treatment discussed in the beginning of the chapter and in the treatment of recurrent disease depicted above are indeed very encouraging. Very recently, new research has indicated that in cell lines and mouse models, PARP inhibitors have immunomodulational properties regardless of BRCAness, which might explain their success in *BRCA*- and HR-negative EOC patients (Shen et al., 2019). Future challenges in terms of PARPi include improvement in our ability to identify ideal candidates for PARPi as well as identification and targeting the mechanisms of drug resistance, and finding novel approaches for combinational therapies (Taylor and Eskander, 2018).

Table 4. NOVA, SOLO2 and ARIEL3 trials.

Trial	Patients	Criteria	Primary endpoint	Control regimen	Experimental regimen	Survival in placebo (months)	Survival in experimental arm (months)
NOVA	553	g*BRCA* mut non-g*BRCA* mut platinum sensitive OC 2 prior platinum regimens	PFS	placebo	niraparib 300 mg daily	g*BRCA* cohort 5,5 non-g*BRCA* cohort 3,8	g*BRCA* cohort 21 non-g*BRCA* cohort 12,9
SOLO2	295	*BRCA 1/2* mutation platinum sensitive OC 2 prior platinum regimens	PFS	placebo	olaparib 300 mg daily	5,5	19,1
ARIEL 3	564	platinum sensitive OC *BRCA*, HRD status 2 prior platinum regimens	PFS	placebo	rucaparib 600 mg twice daily	*BRCA* mut 5,4 HRD 5,4	*BRCA* mut 16,6 HRD 10,8

2.9 HGSC and research prospects

Although chemotherapy and surgery are the standard treatment for ovarian cancer, with results benefiting up to 70% of patients depending on stage and the success of surgery, the disease of the majority of patients with advanced HGSC remains incurable with current options. Basic research is needed to identify underlying molecular mechanisms in patients developing chemoresistant disease, but resistance to new targeted treatments (PARP) is an emerging issue (Jiang et al., 2019). PARP affects many functions in a cell, and although extensive work has been published, there are still areas that need to be explored. The signaling pathways affecting treatment response are an important target of research in the HGSC setting, with possible implications for emerging new treatment modalities such as gene therapies, immunomodulation and combinational targeted therapies.

3 AIMS OF THE STUDY

The aim of this dissertation was to seek insight into early development processes in HGSC, to find possible biomarkers for targeted therapies and to identify biomarkers related to the development of chemoresistance in HGSC. The specific aims for the studies were as follows:

1. To identify early pathogenetic events in HGSC development using microarray technology providing high-throughput screening of genome-wide gene expression in prospectively collected RRSO specimens derived from *BRCA1/2* mutation carriers.

2. To investigate PARP activity measurement and PARP protein expression by IHC in HGSC and their correlation to platinum sensitivity and clinical outcome. Also, the aim was to assess correlation between the two measurement modalities.

3. To identify candidate genes and their respective molecular pathways involved in the pathogenesis and chemoresistance of HGSC.

4 PATIENTS, MATERIALS AND METHODS

All gene and protein names and symbols that appear in this dissertation follow the nomenclature guidelines of the HUGO Gene Nomenclature Committee (Povey et al., 2001; Wain et al., 2002).

4.1 Study subjects and tissue samples

4.1.1 *BRCA* cohort (I)

Nine *BRCA1/2* mutation carriers undergoing RRSO and five control patients with no known *BRCA1/2* mutation undergoing BSO for benign indications were the prospective study cohort and control group, respectively. All tissue samples were taken from macroscopically healthy-looking ovary and distal Fallopian tube.

BRCA1/2 mutation carriers at the time of RRSO surgery were clinically characterized as follows: mean age was 55 years; seven out of nine (78%) patients out of the study group had been diagnosed with breast cancer. The control cohort patients' mean age at the time of surgery was 47 years and they had no history (personal or family) of any gynecological malignancy.

From each *BRCA1/2* mutation carrier, both distal Fallopian tube and ovarian samples were collected. A total of eighteen mutation-positive samples were obtained. In addition, six adnexal control samples were used. For morphological and histopathological findings, the samples were analyzed by an experienced pathologist at the Tampere University Hospital (TAUH) Department of Pathology. In addition, fresh tumor samples from three HGSC patients operated on in TAUH during the study period were obtained and used as additional controls in the study I.

In detail, 5 x 5 x 5 mm-sized tissue samples of distal Fallopian tube and ovarian surface were collected intraoperatively and immediately snap-frozen with liquid nitrogen and stored at -70 °C.

4.1.2 EOC cohort (II, III)

In this prospective study for the assessment of PARP activity in HGSC, one hundred consecutive women providing informed consent and scheduled either for PDS or IDS for suspected or histologically verified OC were recruited at Tampere University Hospital in 2011-2013. Perioperatively, two fresh samples sized approximately 5 x 5 x 5 mm were collected from macroscopically evaluated tumor tissue from each enrolled patient. The samples were taken in the operation room and were immediately snap-frozen with liquid nitrogen and stored at -70 °C. In the case of IDS, fresh tumor samples were collected from the macroscopically visible residual tumor as assessed by an experienced gynecological oncologist.

The final histopathological diagnosis was made by experienced pathologists as part of routine diagnostics at the Department of Pathology in Tampere University Hospital. Finally, only samples from patients with histologically verified primary EOC were included in the study cohort for further analyses of PARP concentration, resulting in a final study cohort of 57 women. From the one hundred originally recruited patients only patients with final diagnosis of ovarian cancer were included and the patients with other diagnoses were excluded.

For the microarray analysis of study III, a subgroup of 12 HGSC patients was chosen from the prospective cohort based on the level of PARP activity. Six patients with the lowest and six patients with the highest PARP levels were included in the microarray analyses.

For all cohorts, clinical and pathological data were collected from the patient records (including age at diagnosis, date of primary or interval surgery, FIGO stage, grade and histological tumor type). Stage was confirmed by a thorough review of the operative and pathology reports by a multidisciplinary team consisting of gynecological oncologists, pathologists and oncologists.

Data regarding chemotherapy were obtained: number of treatment cycles, platinum sensitivity defined as no recurrence within 12 months after the completion of first-line platinum-based chemotherapy, and use of neoadjuvant chemotherapy. For survival analyses, follow-up data were collected from medical records by documenting the time of recurrence or the last follow-up visit or death.

The characteristics of the study patients were as follows: mean age of the patients was 66 years (SD 9.3). Most of the samples were high-grade (70%) and had serous (84%) histology. The median follow-up time was 31 months (range 2-50 months). The characteristics of OC patients included in studies II and III are described in Table 5.

Table 5. Characteristics of the study patients in study II and III.

Study patients' characteristics	n=53 (II)	n=12 (III)
Age at surgery, yrs.	66	65
Median follow-up, months (range)	31 (2-50)	31 (2-50)
Response to platinum therapy[3], n (%)		
Sensitive	38 (71,5)	6 (50)
Resistant	15 (28.5)	6 (50)
FIGO[1] stage, n (%)		
Stage 1-2	5 (9.5)	0 (0)
Stage 3-4	48 (90.5)	12 (100)
Histology, n (%)		
Serous, HGSC	46 (87)	12 (100)
Endometroid	4 (7.5)	0
Papillary	2 (4)	0
Transitional cell	1 (1.5)	0
Grade, n (%)		
Low Grade	10 (19)	0
High Grade	43 (81)	12 (100)
Neoadjuvant therapy, n (%)	19 (36)	5 (42)
Recurrence, n (%)	36 (68)	10 (83)
Death, n (%)	22 (42)	5 (42)

Sensitivity defined as relapse or event-free interval > 12 months after completion of platinum based 1st line therapy.

NACT - neoadjuvant therapy

FIGO - International Federation of Gynecology and Obstetrics

4.2 Methods

4.2.1 RNA extraction (I-III)

For extraction of RNA, approx. 30 mg of fresh frozen ovarian cancer tissue was used. Extraction was performed with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quality and integrity of the RNA were assessed using Fragment Analyzer parallel capillary electrophoresis

(Advanced Analytical Technologies, Ankeny, IA, USA) prior to microarray analysis. Only samples with high-quality RNA with RIN values above 8 were used for analyses. Samples with an RNA concentration minimum of 100 ng/µl were used for gene expression analysis.

4.2.2 Microarray (I; III)

For study I, to generate purified sense-strand cDNA (with incorporated 2´-deoxyuridine, 5´-triphosphate, dUTP), the Ambion WT Expression Kit (Thermo Fischer Scientific, Waltham, MA, USA) was used. For single-stranded cDNA synthesis (sscDNA), 15 µg of cRNA was taken, and DNA was labeled by terminal deoxynucleotidyl transferase (TdT) with the Affymetrix® proprietary DNA Labeling Reagent. This agent was then covalently linked to biotin using the Affymetrix GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA). Subsequently, 5.5 µg of sscDNA was fragmented and hybridized to the GeneChip array (Affymetrix Human Gene 1.0 ST Array) in cartridge format during a 17-h incubation at 45 °C. After hybridization, the array was washed and stained with streptavidin phycoerythrin conjugate using an automated protocol on a GeneChip® Fluidics Station 450, followed by scanning on a GeneChip® Scanner (Affymetrix Human Gene 1.0 ST Array).

For study III, the Agilent gene expression microarray kit (Agilent Technologies, Santa Clara, CA, USA) was used for RNA extraction as described above and then labeled and hybridized according to the manufacturer's instruction. RNA was labeled with Cy3 fluorochrome and thereafter co-hybridized for 21 h in Agilent 4X44K human gene expression array slides, using Xpress Ref TM Human Universal Reference Total RNA (SuperArray Bioscience Corporation, Frederick, MD, USA) labeled with Cy5 as a control. The slides were scanned on an Agilent C scanner. The raw data obtained were then extracted using Agilent Feature Extraction software ver. 11.0.1.1 and quantile normalized.

4.2.3 Quantitative Real-Time PCR (qRT-PCR) (I, III)

The validation of microarray data in both studies was performed by qRT-PCR as previously described (Balacescu et al., 2014).

For study I, six genes were chosen for qRT-PCR validation based on fold change, signal intensity, rank in the list of differentially expressed genes and possible presence

in gene ontology categories. The chosen genes were *KLF4, PLK, SIK1, TDP2, AQP9* and *EGR3*. A total of 385 ng of RNA was reverse-transcribed using the DyNAmo cDNA Syndissertation Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The primer and probe sequences are as follows:

KLF4 fwd.	CCATCTTTCTCCACGTTCG
KLF4 rev	AGTCGCTTCATGTGGGAGAG
PLK3 fwd.	CAGCACCTGAAGACAGCTCA
PLK3 rev	AGAGCACAAAGGGCTGACTC
SIK1 fwd.	GGGACCTCAAGACCGAGAA
SIK1 rev	GAGGCTCTCCTGACTTGTAGAAA
TDP2 fwd.	TCGAGGGGTGTGTTCCTACT
TDP2 rev	TGGGGGAATAACTTCCTGTAGA
AQP9 fwd.	GCAACCGTCTTTGGCATTTA
AQP9 rev	TTTTCTCCCACGATCAGCA
EGR3 fwd.	CAATCTGTACCCCGAGGAGA
EGR3 rev	CAGACCGATGTCCATTACATTC

For study III, qRT-PCR validation was based on fold change, q-value, signal intensity and literature data. The 14 putatively differentially expressed genes were *ROR2, GREB1, CAST, ATP6V1D, TOP1MT, TMOD1, MYCN, DLK1, PLEKHG4B, B4GALNT4, SLC35F3, PTCH2, TNNC1*, and *BNC1*. mRNA of the chosen genes was reverse-transcribed using random hexamer primers and MultiScribe reverse transcriptase (Thermo Fischer Scientific, Waltham, MA, USA). Thereafter, Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fischer Scientific, Waltham, MA, USA) on a BioRad CFX96 ™ Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA, USA) was used for qRT-PCR. Each sample was run in duplicate. Furthermore, expression values were normalized against the TATA-binding protein (TBP), which is a traditional housekeeping gene often used for normalization purposes because of its stability (Gao et al., 2019; Macabelli et al., 2014). The primer sequences for the validated genes are detailed below:

GREB1 fwd.	5'ATGGGAAATTCTTACGCTGGAC
GREB1 rev	5'CACTCGGCTACCACCTTCT
ROR2 fwd.	5'GTGCGGTGGCTAAAGAATGAT
ROR2 rev	5'ATTCGCAGTCGTGAACCATATT
CAST fwd.	5'CAAGCCGGGTGACAAGAAAAA
CAST rev	5'CCCGATGGTTTATCCGGTTTAG
ATP6V1D fwd.	5'AGCAGGTGTTACTTTGCCAGT

ATP6V1D rev	5'AGTTCCACCAGTAGTTCCACT
TOP1MT fwd.	5'ACGAAGACGGGGTGAAGTG
TOP1MT rev	5'CCGGAAAACCTCCTTTGTTGTG
TMOD1 fwd.	5'ATGTCGTACAGACGAGAACTAGA
TMOD1 rev	5'CAGTGCATTATCAGGGTCCAG
MYCN fwd.	5'ACCCGGACGAAGATGACTTCT
MYCN rev	5'CAGCTCGTTCTCAAGCAGCAT
DLK1 fwd.	5'CTTTCGGCCACAGCACCTAT
DLK1 rev	5'TGTCATCCTCGCAGAATCCAT
PLEKHG4B fwd.	5'AAGCGGTTGGAGTTGGATGAC
PLEKHG4B rev	5'CACGATCTGGAGCAGTAGGTG
B4GALNT4 fwd.	5'GCTGCCACTGAACTTCACC
B4GALNT4 rev	5'TGCGCGTATGAGGGAACAG
SLC35F3 fwd.	5'GTGAAGCAGCGATACAGGGAA
SLC35F3 rev	5'GCAGAACAACACGGAGACATC
PTCH2 fwd.	5'GCTTCGTGCTTACTTCCAGGG
PTCH2 rev	5'CATGCGGAGACCTAATGCCA
TNNC1 fwd.	5'TGGTTCGGTGCATGAAGGAC
TNNC1 rev	5'GTCGATGTAGCCATCAGCATT
BNC1 fwd.	5'GCTGCAAGATCCCCTACGAAG
BNC1 rev	5'ACGAAGAAGTGTACGAAGGGTT
TBP fwd.	5'GAATATAATCCCAAGCGGTTTG
TBP rev	5'ACTTCACATCACAGCTCCCC

4.2.4 PARP concentration by ELISA pharmacodynamic assay (II)

The PARP (poly(ADP-ribose)) concentration was measured using the HT PARP in vivo Pharmacodynamic Assay II by Trevigen (Gaithersburg, MD, USA) according to the manufacturer's instructions and as described in detail in the original publication. Briefly, approx. 10 mg of frozen tumor tissue was homogenized and subsequently sonicated (Sonics, Vibra-Cell, Newtown, CT, USA). Sodium dodecyl sulfate (SDS) was added to the lysates to a final concentration of 1.0%, and samples were incubated at 100 °C for 5 min. Supernatants were collected, and equal amounts (approx. 3 μg) of protein lysates were analyzed in duplicate with an ELISA assay; PARP concentrations were quantified based on chemiluminescent signal measurement (Luminoskan Ascent, Thermo Fisher Scientific, MA, USA). Commercial protein lysate controls were included in the assay.

4.2.5 Immunohistochemistry (II)

In study II, PARP-1 protein expression was evaluated by immunohistochemistry (IHC). For this, the Department of Pathology at Tampere University Hospital

provided representative archival ovarian cancer samples. A standard microtome was used to cut the tumor sample blocks into 3-4 μm-thick sections. Subsequently, the slides were deparaffinized and rehydrated (in graded ethanol). Pretreatment was performed with a PT-Module (Lab Vision, Fremont, CA) at 98 °C for 15 min in 0.05 M TrisHCl buffer, pH 9.0, containing 0.001 M EDTA, as described in the original publication. The primary PARP-1 antibody (anti-PARP-1, Cat# sc-8007; Santa Cruz Biotechnology, Dallas, TX) was used at a dilution of 1:5,000. Visualization of PARP-1 was carried out with a PowerVision+ polymer kit (Leica Biosystems Newcastle Ltd., Newcastle, UK), and diaminobenzidine was used as a chromogen (DABImmPact, Vectorlabs, Burlingame, CA). Counterstaining with hematoxylin (Mayer's hematoxylin, Oy FFChemicals Ab, Haukipudas, Finland) was performed, and slides were then dehydrated, cleared and mounted. Autostainer 480 (Lab Vision, Los Angeles, CA, USA) was used for staining protocols. Placenta was used for a positive tissue control. In each staining batch, positive and negative (primary antibody omitted) controls were included. An Olympus System Microscope BX43 was used to analyze the samples; staining was assessed by two independent researchers. The researchers were blinded with regard to the clinico-pathological information of the patients and PARP concentration determined by ELISA pharmacodynamic assay. Semiquantitative scoring was used for PARP-1 nuclear staining intensity: low (negative or weak nuclear staining), intermediate (more pronounced or focally more intensive nuclear staining compared to the low staining pattern) or strong (intensive uniform nuclear staining).

4.2.6 Western blotting (III)

In study III, protein assessment was performed as follows: frozen tumor pieces were thawed, washed twice with cold PBS and incubated in lysis buffer containing 50 mM Tris-HCl pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton-x-100, 50 mM NaF supplemented with protease and phosphatase inhibitor cocktails (Bimake, Houston, TX, USA). Prior to SDS-PAGE gel electrophoresis and Western blotting, the lysates were mixed with 4X Laemmli loading buffer. The details of the primary antibodies are shown below in Table 6:

Table 6. Primary antibodies in Western blotting.

Akt S473	(#4060)
pMEK1/2 S217/221	(#9121)
NF-κB p65	(#6956)
pPI3K p85 Y458/p55 Y199	(#4228)
PI3K p85α	(#13666)
Rac-1	(#4651)
pSTAT3 Y705	(#9145)
STAT3	(#9139)
Wnt5a/b	(#2530) (Cell Signaling Technology, Danvers, MA, USA)
Akt	(#sc-5298)
Bcl-2	(#sc-7382)
MEK1/2	(#sc-6250)
β-tubulin	(#sc-166729) (Santa Cruz, Dallas, TX, USA)
ROR1 6D4	(Dr. Riddel lab)
ROR2	(#565550) (BD Biosciences, San Jose, CA, USA)

IRDye® 800CW donkey anti-mouse IgG or IRDye® 680RD donkey anti-rabbit IgG (LI-COR, Lincoln, NE, USA) was used as the secondary antibody. Odyssey and LiCor software were used for scanning and quantifying the blots. The β-tubulin expression level was used to normalize protein expression levels in the Western blot assays.

4.2.7 Cell culture (III)

For cell line experiments, cells were purchased from Merck & Company Inc. (Kenilworth, NJ, USA), and the A2780 and A2780cis cell lines were cultured according to the manufacturer's recommendations. Medium containing 1 μM cisplatin (Selleckchem, Munich, Germany) was used to maintain the A2780 line. The cisplatin EC50 responses of A2780 and A2780cis cells were validated by incubating cells with increasing concentrations of cisplatin for 3 days. CellTiterGlo (CTG) Assay (Promega, San Luis Obispo, CA, USA) was used to investigate cell viability.

Subsequently, TRI Reagent® (Molecular Research Center Inc. Cincinnati, OH, USA) was used to collect RNA from A2780 and A2780cis according to the manufacturer's protocol, and qRT-PCR was performed as described in the original publication and section 4.2.3 of this dissertation. The expression of ROR2 and GREB1 was normalized against TBP, and each cell line was run in 4 replicates.

4.2.8 Statistical analysis (I-III)

In studies I and III, qRT-PCR data were analyzed using the Student's t-test and Mann-Whitney U test where appropriate, and Kaplan-Meier and Cox regression analyses were used to estimate the survival rates from the date of surgery (PDS) or from the date of the first dose of NACT until the date of the event of interest. For PFS, the event of interest was a recurrence or death, whichever occurred first. Patients alive at the last follow-up without a recurrence were censored at the last follow-up date. Statistical analysis was performed using GraphPad Prism 6 software for Windows (GraphPad Software Inc., La Jolla, CA, USA) for study III and version 22 of IBM SPSS statistics software (IBM Inc., Armonk, NY, USA) for studies I and II. In all studies, a p-value less than 0.05 was considered significant. For gene expression and survival data, the Oncomine (https://www.oncomine.org/resource/login.html) and Kaplan-Meier plotter (http://kmplot.com/analysis/index.php?p=service&cancer=ovar) databases were searched.

4.2.8.1 Bioinformatics (I)

GO Slim ontology was used for Gene Ontology (GO) enrichment analysis (Davey et al. 2011). GO human annotation files (ver. 6/11/2013) and experimentally validated interactions (EXP, IDA and IPI) were also used. To test the significance of the enrichment, Bonferroni correction testing was implemented. The significance level threshold was set at 0.05. In MATLAB® (R2012b), GO analysis was performed using the Bioinformatics Toolbox® functions to handle the GO structure.

4.2.8.2 Microarray data analysis (I, III)

In study I, remapped gene annotations from the Brainarray Custom CDF files (HuGene10stv1_Hs_ENSG, v.14.1.0) (Gentleman et al., 2004) were used for GeneChip® signal intensity processing with robust multiarray analysis. Simpleaffy, limma, BioMart, and qvalue packages of the Bioconductor project (Storey and Tibshirani, 2003; Gentleman et al., 2004; Smyth, 2004; Durinck et al., 2005; Wilson and Miller, 2005) were used for analysis that was implemented in R (the R project for Statistical Computing). Differential expression significance was assessed using the empirical Bayes moderated paired t-statistics followed by p-value adjustment with the FDR (false discovery rate) approach (Smyth, 2004). Before statistical testing, arrays were quality weighted (Ritchie et al., 2006). Differentially expressed genes were considered to be genes with FDR-corrected P-values ≤ 0.05. Hierarchical clustering (HC) and principal component analysis (PCA) were used for data visualization in MATLAB® (R2012b). The raw gene expression data have been uploaded into the GEO website (http://www.ncbi.nlm.nih.gov/geo/).

In study III, implementation in R using the packages limma and preprocess Core of Bioconductor project (Gentleman et al., 2004b; Bolstad et al., 2003; Smyth, 2004) was performed for the statistical analysis of mRNA microarray data. The probes with the highest average expression were chosen, and the data were quantile-normalized (Bolstad et al., 2003). Differential expression was identified between patients who had low PARP value/platinum resistance and patients who had high PARP/platinum sensitivity using the limma approach (Smyth, 2004). The empirical Bayes moderated t-test was used to obtain P-values. To determine differential mRNA expression, a fold-change cutoff of 2 was used. Principal component analysis and hierarchical clustering were used to interpret the results (Mardia et al., 1992).

4.3 Ethical aspects (I-III)

In conducting the studies, the Declaration of Helsinki was followed. The protocols of studies I-III were approved by the Pirkanmaa Hospital District Ethics Committee. All study and control patients provided informed consent to express their willingness to participate in the study. Prior to consenting, patients had received a written patient information leaflet, and they had sufficient time to read it and an opportunity to ask additional questions regarding the study. The patients were informed in accordance with TENK guidelines (http://www.tenk.fi/fi/tenkin-ohjeistot) that at any point of

time they can withdraw their consent. Patients were also informed that participation in the study does not affect the treatment of their medical condition, nor would study participation subject the patients to any additional intervention.

5 RESULTS

5.1 Differentially expressed genes in samples from RRSO (I)

5.1.1 Expression array

A total of 454 transcripts were identified from 22,733 genes (all ovarian and Fallopian tube samples were pooled together) as differentially expressed in samples from *BRCA1/2* mutation carriers relative to controls. Two hundred ninety-nine genes were downregulated, and 155 genes were upregulated; the 20 most down- and upregulated genes are presented in Table 7. In addition, when only *BRCA1/2*-mutation-positive Fallopian tube samples were compared with controls, 148 transcripts were expressed differentially. Of these, 34 genes were upregulated, and 114 were downregulated.

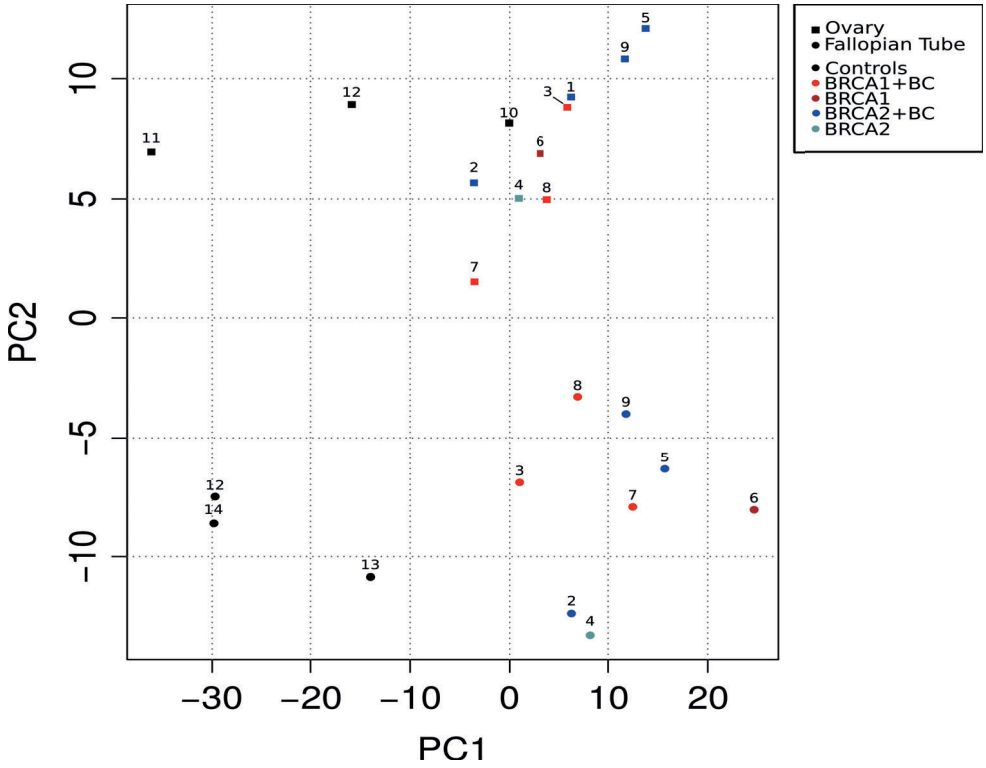
Unsupervised clustering was used to analyze global gene expression patterns. This analysis separated ovarian and Fallopian tube samples from each other as expected and, furthermore, controls from *BRCA* mutation carriers. Interestingly, *BRCA1/2*-mutation-positive samples mostly grouped together regardless of personal history of breast cancer. The results of unsupervised clustering are visualized in Figure 8.

Table 7. The 20 Most Up- and Downregulated Genes in *BRCA1/2* Ovarian and Fallopian Tube RRSO* Samples.

Gene name	Gene description	Fold change	P-value	Gene name	Gene description	Fold change	P-value
U4	U4 small nuclear RNA	3,21	0,011	*MIR22HG*	MIR22 host gene	-3,55	0,005
NIPAL3	NIPA-like domain containing 3 family members	2,65	0,027	*KLF4*	Kruppel-like factor 4	-3,6	0,0004
AQP9	Aquaporin 9	2,22	0,046	*HBEGF*	Heparin-binding EGF-like growth factor	-3,72	0,003
PLAG1	Pleiomorphic adenoma gene 1	2,1	0,028	*ATP1B3*	ATPase, Na+/K+ transporting, beta 3 polypeptide	-3,83	0,003
RPS26P3	Ribosomal protein S26 pseudogene 3	2,09	0,046	*MT1A*	Metallothionein 1A	-3,84	0,005
C5orf54	Chromosome 5 open reading frame 54	2,08	0,014	*ZNF331*	Zinc finger protein 33	-3,86	0,005
SLC9A11	Solute carrier family 9, member 11	1,99	0,035	*SLC2A3*	Solute carrier family 2	-3,89	0,001
CCL28	Chemokine (C-C motif) ligand 28	1,85	0,008	*NR4A1*	A member of nuclear receptor family, involved in cell death	-3,98	0,005
KRT8	Keratin 8	1,84	0,049	*MT1M*	Metallothionein 1M	-4,02	0,013
ZNF772	Zinc finger protein 772	1,77	0,007	*CYR61*	Cysteine-rich, angiogenic inducer, 61	-4,09	0,039
ZNF232	Zinc finger protein 232	1,73	0,005	*CSRNP1*	Cysteine-serine-rich nuclear protein 1	-4,09	0,001
GALM	Galactose mutarotase	1,71	0,031	*ZFP36*	Zinc finger protein 36	-4,14	0,003
SCARNA15	Small Cajal body-specific RNA 1	1,69	0,046	*NR4A2*	NR4A2 nuclear receptor subfamily 4, group A, member 2	-4,5	0,012
WDR5B	WD repeat domain 5B	1,66	0,01	*SOCS3*	Suppressor of cytokine signaling 3	-4,58	0,0004
P2RY13	Purinergic receptor P2Y, G-protein coupled, 13	1,66	0,015	*ADAMTS4*	ADAM metallopeptidase with thrombospondin type 1 motif	-4,85	0,0008
THNSL2	Threonine synthase-like 2 (S. Cerevisiae)	1,65	0,0104	*CXCL2*	Chemokine (C-X-C motif) ligand 2	-4,87	0,003
DPY19L2P	Dpy-19-like 2 pseudogene 2 (C. elegans)	1,65	0,015	*SIK1*	Salt-inducible kinase1	-4,93	0,001
Y_RNA	Y RNA	1,63	0,046	*NR4A3*	Nuclear receptor subfamily 4, group A, member 3	-5	0,003
DENND2D	DENN/MADD, a signaling protein that has multiple functions	1,62	0,041	*EGR3*	Early growth response 3	-5,43	0,003
MCTP2	Multiple C2 domains, transmembrane 2	1,57	0,046	*FOSB*	FBJ murine osteosarcoma viral oncogene homolog B	-7,31	0,002

*RRSO, Risk-reducing salpingo-oophorectomy

Figure 8. Scatterplot showing location of samples (control, *BRCA1* and *BRCA2* ovarian and Fallopian tube) along the first two principal components (PC1 and PC2). Squares: ovarian samples; dots: Fallopian tube samples. Black: control samples, bright and dark red: samples from *BRCA1* mutation carriers with or without a personal history of breast cancer, respectively. Cyan and blue color: samples from *BRCA2* mutation carriers with or without a personal history of breast cancer, respectively.



5.1.2 qRT-PCR

KLF4, PLK, SIK1, TDP2, AQP9 and *EGR3*, the six differentially expressed genes in the microarray analysis, were selected for validation by qRT-PCR in the following 10 samples: 4 *BRCA1/2*-mutation positive, 3 control, and in 3 HGSC samples. As a result, the genes showed similar expression patterns in the qRT-PCR validation as those in the original microarray, which further validates the microarray results as intended. Comparable patterns of expression were observed in five out of six genes in *BRCA1*/2-mutation-positive RRSO and HGSC samples. qRT-PCR analyses are summarized in Table 8.

Table 8. Differentially expressed genes selected for validation by quantitative RT-PCR in *BRCA1/2* RRSO, serous ovarian carcinoma and control samples.

Gene	Expression in ***BRCA1/2*** ovarian and Fallopian tube samples (n=4)[1]	Expression in serous ovarian carcinoma samples (n=3)[1]	Expression in original array
KLF4a	0.48	0.26	3.4x downregulation
PLK3b	0.69	1.28	2.0x downregulation
SIK1c	0.16	0.07	4.9x downregulation
TDP2d	1.83	2.23	1.5x upregulation
AQP9e	6.32	2.12	2.2x upregulation
EGR3f	0.12	0.08	5.4x downregulation

[1] Samples from normal ovarian and Fallopian tube tissue from mutation-negative patients (n=4); in quantitative RT-PCR analyses relative expression levels of selected genes in the control samples was set to 1.00 to which gene expression in *BRCA1/2* RRSO and serous ovarian cancer samples was then compared.

a Kruppel-like factor 4

b Polo-like kinase 3

c Salt-inducible kinase 1

d Tyrosyl DNA phosphodiesterase-2

e Aqua-glyceroporins 9

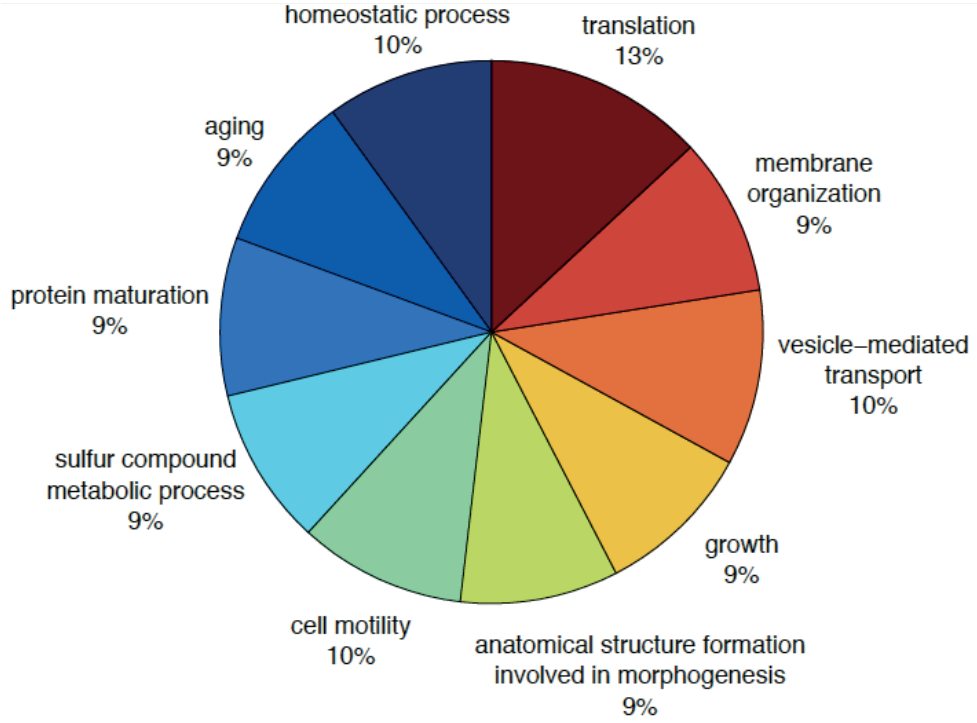
f Early growth response 3.

GO=gene ontology; RRSO= Risk-reducing salpingo-oophorectomy

5.1.3 Gene ontology enrichment analysis

To identify biological processes in which the identified differentially expressed genes are involved, GO enrichment analysis was performed. A significant p-value was observed for ten different GO terms, and these are summarized in Figure 9. In addition, ten enriched biological processes were identified when one hundred overlapping differentially expressed genes were analyzed from ovarian and Fallopian tube versus control samples and Fallopian tube versus control samples (Figure 9).

Figure 9. Enriched biological processes in differentially expressed genes shared between ovarian and Fallopian tube versus control samples and Fallopian tube versus control samples.



GO Term	p-val	counts (DE / BG)	definition
GO:0042592	4.08e-06	19 / 1157	homeostatic process
GO:0007568	7.20e-06	18 / 1097	aging
GO:0051604	8.74e-06	18 / 1112	protein maturation
GO:0006790	1.06e-05	18 / 1127	sulfur compound metabolic process
GO:0048870	1.06e-05	19 / 1234	cell motility
GO:0048646	1.15e-05	18 / 1134	anatomical structure formation involved in morphogenesis
GO:0040007	1.41e-05	18 / 1150	growth
GO:0016192	1.73e-05	20 / 1387	vesicle-mediated transport
GO:0061024	1.77e-05	18 / 1169	membrane organization
GO:0006412	2.16e-05	25 / 2003	translation
Total D.E.:	100		
Total genes:	18181		
Unique gene names:	16697		

5.2 PARP expression (II)

5.2.1 PARP concentration by ELISA pharmacodynamic assay

In 51 out of 57 study tumors, PARP levels were measurable (89%) and ranged between 0.67- 1024.68 pg/ml. The median and mean values were 203 and 311.1 (SD 292.1) pg/ml. Corresponding to the median value, the high PARP activity cut-off level was set to 203 pg/ml.

As a main result, we found that a high PARP concentration was associated with platinum sensitivity in the entire study cohort (p=0.022), and this result was prominent in the high-grade subgroup (p=0.017). However, low levels of PARP concentration were associated with NACT (p=0.014).

5.2.2 PARP-1 immunohistochemistry

Fifty-six representative tumor blocks were available for PARP-1 staining analyses. Half (or 28) of the samples showed low/moderate PARP nuclear staining. In turn, another half showed strong staining. PARP concentration determined by pharmacodynamic assay was not associated with IHC PARP-1 staining. Low PARP-1 IHC staining was associated with normalized serum CA12-5 levels after three cycles of platinum-based chemotherapy (p=0.027).

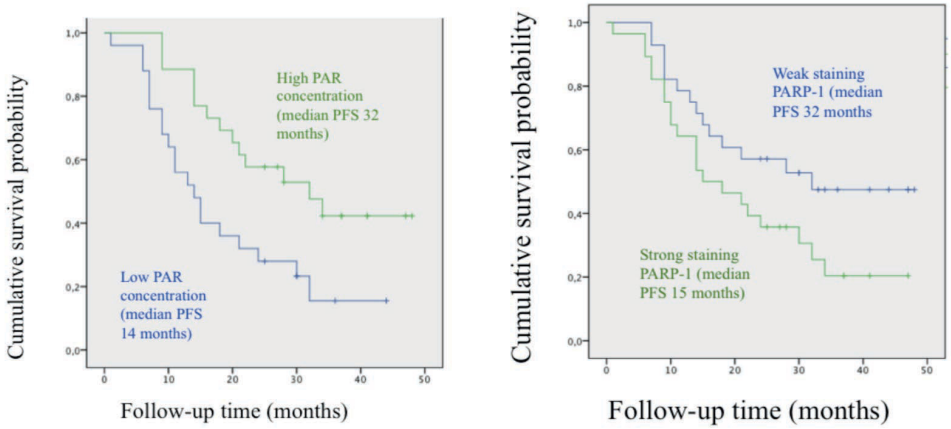
5.2.3 Survival analyses

Longer PFS was associated with a high PARP concentration when compared to a low concentration (32 vs 14 months, respectively, log-rank p=0.009; Figure 10a). A low PARP concentration was associated with an increased risk of recurrence both in univariate (HR 2.4; 95% CI 1.204-4.797) and multivariate analyses using Cox regression. There was no association with increased risk of death (HR 1.12; 95% CI 0.455-2.764).

Additionally, a trend for the association of longer PFS with low PARP-1 by IHC was detected (32 vs 15 months, log-rank p=0.061; Figure 10b). However, low PARP-1 IHC was not associated with decreased risk of recurrence in multivariate Cox regression analysis. There was also no association with low PARP-1 IHC staining and overall survival (HR 0.724; 95% CI 0.307-1.708).

Figure 10. a. Kaplan-Meier analysis of progression-free survival (PFS) according to median level of PARP concentration (log-rank p =0.009). The cut-off level for high PARP concentration was set to 203 pg/ml (median value of PARP concentration). Vertical lines represent censored patients.

b. Kaplan-Meier analysis of progression-free survival (PFS) according to PARP-1 immunohistochemistry staining (log-rank p =0.061). Vertical lines represent censored patients.



5.3 Gene expression analyses (III)

5.3.1 Microarray analysis of a subgroup of HGSC patient samples based on platinum sensitivity and PARP activity

To evaluate gene expression in HGSC samples targeting the genes possibly associated with chemoresistance, a subgroup of samples with platinum sensitivity and high PARP activity (defined in the analyses of study II) was compared to samples with platinum resistance and low PARP activity (n=12). The genome-wide microarray analysis revealed a total of 3,001 differentially expressed genes between the two comparison groups when a log fold change cut-off of 2 was implemented. Altogether, 1463 downregulated genes and 1538 upregulated genes were identified.

5.3.2 Differential expression of *ROR2* and *GREB1*

Fourteen differentially expressed mRNAs identified in the microarray analysis (*ROR2, CAST, ATP6V1D, GUCY1A3, TMOD1, MYCN, DLK1, PLEKHG4B, GREB1, B4GALNT4, SLC35F3, PTCH2, TNNC1, BNC1*) were selected for validation by qRT-PCR in the original cohort of 53 ovarian cancer patients. qRT-PCR validation confirmed two differentially expressed genes: *ROR2* and *GREB*1. These two genes were differentially expressed in high PARP/platinum-sensitive vs low PARP/platinum-resistant group of EOC samples (for *ROR2* p=0.02 and for *GREB1* p=0.002). In high PARP/platinum-sensitive tumor samples, *ROR2* was downregulated, and *GREB1* was upregulated.

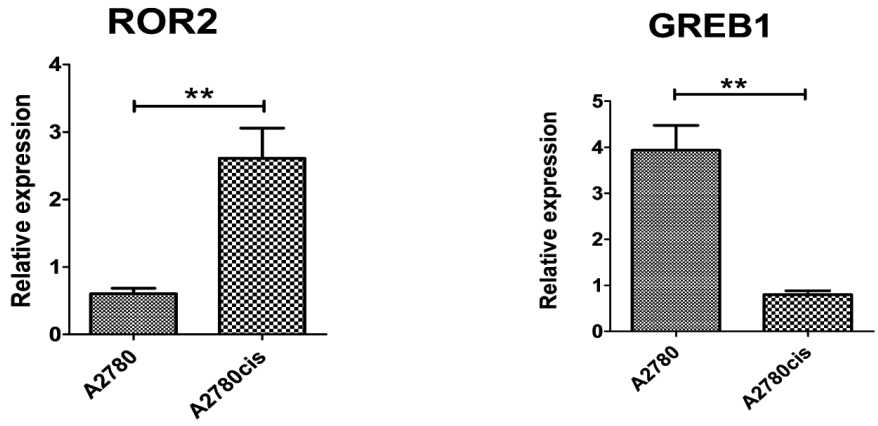
5.3.3 Analyses of ROR2 signaling pathway

To further investigate ROR2 expression in the setting of low PARP activity/platinum-resistance, the protein expression levels of Wnt5a (ROR1 and ROR2 ligand), ROR1 and ROR2 were examined by Western blot in a subcohort of samples (n=30) that were chemonaïve (only cases with PDS were included). In the low PARP/platinum-resistant subgroup, Wnt5a, the ROR1 and ROR2 proteins had higher expression levels. In addition, downstream signaling mediators such as pSTAT3 (Y705) and NF-kB were also upregulated in the tumor samples with platinum resistance.

5.3.4 ROR2 and GREB1 expression in cisplatin-resistant cell line model

The A2780 and cisplatin-resistant A2780cis ovarian cancer cell lines were chosen to further investigate the expression levels of ROR2 and GREB1. Cisplatin resistance of A2780cis cells compared to that of A2780 parental cells was confirmed by CTG assay. ROR2 mRNA upregulation in the platinum-resistant A2780cis cell line compared to the platinum-sensitive A2780 cell line was observed (p=0.0046; Figure 11). In addition, GREB1 mRNA levels were downregulated in A2780cis relative to A2780 parental cells (p=0.0012; Figure 11).

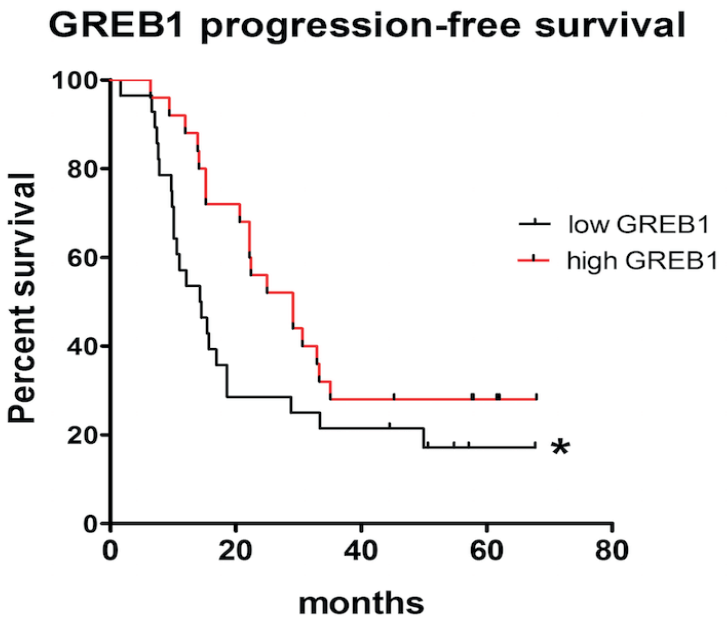
Figure 11. ROR2 and GREB1 mRNA expression in platinum sensitive and resistant cell lines.



5.3.5 Survival analyses with respect to *GREB1* and *ROR* expression

Association of *GREB1* expression with patient survival was analyzed in the original cohort of 53 ovarian cancer patients. High *GREB1* expression was associated with longer PFS ($p = 0.019$ in log-rank test; Figure 12). Furthermore, a database search was performed in the online Kaplan-Meier plotter database. The online database included data from 1465 ovarian cancer patients and showed a similar result, with high *GREB1* expression associating with a longer PFS (log-rank $p=0.014$). *ROR2* expression levels had no association with patient survival in the original patient cohort. Additionally, no overall or progression-free survival data were available in the online Oncomine or Kaplan-Meier plotter databases with respect to *ROR1/2* expression in EOC.

Figure 12. Progression free survival according to median level of *GREB1* concentration (log rank p= 0.019). Vertical lines represent censored patients.



6 DISCUSSION

6.1 *BRCA*-mutated RRSO cohort vs healthy women (I)

Genome-wide investigations in *BRCA1/2* mutation carriers from RRSO samples remain scarce (Tone et al., 2008; George et al., 2011; Press et al., 2010). *BRCA* mutation carriers' RRSO histopathology reports have been published, underlining the importance of timely procedures in terms of preventing OC (Lee et al., 2017) (Zakhour et al., 2016). Investigation of the Fallopian tube tissue from *BRCA* mutation carriers might reveal insight into the early phases of tumor development. This was the hypothesis for the first study in this dissertation.

To investigate the early pathogenetic events in EOC development, the genome-wide gene expression levels in RRSO samples derived from *BRCA1/2* mutation carriers were studied and compared to those of healthy controls. The microarray assay yielded a variety of differentially expressed genes, which were then evaluated based on their presence in one or more GO categories, and six genes were then chosen for further validation: *KLF4, PLK3, SIK1, TDP2, AQP9*, and *EGR3*. The validation was carried out in HGSC samples that were selected for their positive BRCAness phenotype (Konstantinopoulos et al., 2010).

In detail, the validation results were comparable to the initial microarray expression of all other genes except *PLK3*. This suggests that *KLF4, SIK1, TDP2*, *AQP9*, and *EGR3* may play roles in ovarian carcinogenesis. The *PLK3* expression discrepancy suggests that perhaps it is not involved in the early pathogenesis of OC, although either overexpression or downregulation of PLK proteins occurs frequently in various cancer types (prostate, breast, leukemia, lung) (Maniswami et al., 2018; Archambault et al., 2015).

TDP2 was found to be upregulated in the microarray assay and validation analysis. TDP2 is a DNA-repairing phosphodiesterase that acts by excising topoisomerase II- and potentially also topoisomerase III-DNA adducts. It is also involved in signal transduction (under the former names of TTRAP or EAPII) (Pommier et al., 2014). Additionally, TDP2 suppresses chromosome translocations from DSBs introduced during gene transcription (Gómez-Herreros et al., 2017). The regulation

of TDP2 activity by posttranslational modifications in DNA repair remains unclear (Bian et al., 2016). It has not been previously associated with ovarian cancer.

Furthermore, *EGR3* (early growth response 3) regulates signal transduction, cell growth, and proliferation (Patwardhan et al., 1991) (Blok et al., 1995). In our study, *EGR3* was the second most downregulated gene in the expression array and concordantly was also downregulated in HGSC samples, suggesting a potential role as a tumor suppressor, a finding not published previously. A cancer association has been made in other tumor types: cutaneous squamous cell carcinoma (Wei et al., 2018), leukemia (R. Li et al., 2017) and nonsmall-cell lung cancer, among others (Chien et al., 2017). The mechanism of EGR3 function in tumor development is through the JAK-STAT signaling pathway, affecting the expression of *SOCS3* (suppressor of cytokine signaling 3, also downregulated in our array), a negative regulator in this pathway. Its aberrant function is possibly associated with tumor growth (Inagaki-Ohara et al., 2013).

Another validated gene was *KLF4*, a gene belonging to the Krüppel-like factors (KLFs), which are a family of DNA-binding transcriptional factors linked by a triple zinc finger DNA-binding domain modulating diverse and essential functions in multiple cellular processes, including differentiation, proliferation, migration, pluripotency and inflammation (Tetreault et al., 2013; Jiang et al., 2008). KLF4 can both activate and repress transcription, depending on the contents of target promoters (Zhang et al., 2000), and additionally, KLF4 functions as an oncogene or a tumor suppressor depending on the type of cancer: in intestinal epithelium it acts as a suppressor, and in skin, larynx and breast, it acts an oncogene (Evans and Liu, 2008). It has also been discovered that PARP1 is a KLF4-interacting partner: in cancer and stem cells PARP1 recruits KLF4 to activate telomerase expression and stem cell pluripotency, indicating a positive regulatory role (Hsieh et al., 2017).

It has been previously shown that that KLF4 functions as a tumor suppressor gene in OC by inhibiting TGFβ-induced epithelial-to-mesenchymal transition (Chen et al., 2014). Epithelial-to-mesenchymal transition greatly facilitates the spread of cancer cells and is characterized by the loss of epithelial markers and the acquisition of mesenchymal markers, thus making the cells more migratory and invasive (Zhang et al., 2018). KLF4 depletion modulates OC cells towards being more migratory and invasive. *KLF4* has also been hypothesized being a driver in cisplatin resistance in the setting of EOC cell line model (Lund et al., 2017). However, a result stating the opposite has also been published – in another OC cell line model, *KLF4* expression was found to enhance the efficacy of chemotherapy drugs and the authors proposed that inducing *KLF4* expression with APTO-253

might be a novel therapeutic strategy for treating OC (Wang et al., 2017). Further studies are needed to clarify the role of *KLF4* in EOC.

The *SIK1* gene was also downregulated. The salt-inducible kinases (SIKs) represent a subfamily of AMP-activated protein kinase (AMPK) family kinases; the major biological role of these kinases is to control gene expression in response to extracellular cues that increase intracellular levels of cAMP (Wein et al., 2018). In terms of carcinogenesis, reduced *SIK1* expression is correlated with poor prognosis in two large human breast cancer datasets (Shaw, 2009). SIK1 suppresses metastasis p53-dependently (Cheng et al., 2009). SIK1 also has been suggested to act as a novel biomarker and therapeutic target for lung cancer (Yang et al., 2018). In OC, SIK1, which is also known as MSK/SIK/SNF1LK, has been shown to be downregulated and its expression regulated by miR-141 (Chen et al., 2016). In another study, enhancement of LKB1-SIK1 suppressed the growth and aggressiveness of clinical sample OC cells, leading to an inhibition of metastatic potential (Hong et al., 2018). The findings published previously and presented here strongly suggest a role in OC, although more detailed understanding is impossible without further investigations.

AQP9 belongs to the aquaporins, which are transmembrane water channel proteins involved in transport of fluid, transcellular water movement and cell migration (Ribatti et al., 2014). In this study, *AQP9* was upregulated. AQP9 has previously been associated with several cancers, such as hepatocellular (through FOXO1 expression upregulation) and prostate cancer (through ERK pathway) (Li et al., 2016; Q. Chen et al., 2016). According to previous data, aquaporin expression in tumor cells and vessels facilitates tumor growth and spread, suggesting a possibility for an antitumor therapy (Verkman et al., 2008). In OC, previous studies have demonstrated that there is significantly higher AQP7 and AQP9 protein expression in malignant and borderline tumors than in benign tumor and normal ovarian tissue (Yang et al., 2011). Additionally, in this study, the AQP9 expression level was positively and significantly correlated with tumor grade and histological type. Other aquaporins from the AQP family members have been associated with varied prognostic properties and drug sensitivity regulation in OC (Chen et al., 2015; Chetry et al., 2018).

The study aimed to uncover genes possibly involved in the early pathogenesis of EOC. Some of the genes found to be associated with ovarian cancer have been indicated previously, such as *AQP9, SIK1* and *KLF4*. *TDP2* and *EGR3* have not been previously associated with OC and thus represent a novel finding. It would be of interest to further investigate these genes in OC cell line models, as well as larger cohorts of OC patient-derived samples. Previous RRSO studies in *BRCA* mutation

carriers have also revealed interesting genes possibly associated with OC, although the results are somewhat conflicting. A study from 2008 found *DAB2* and *SKIL* were differentially expressed (Tone et al., 2008), whereas a study from 2010 found *CDKN1C* and *EFEMP1* were differentially expressed (Press et al., 2010). Another RRSO study revealed 440 differentially expressed genes and abrogated pathways (George et al., 2011). None of the results in this study overlap with these previous findings, indicating the heterogeneity of different alterations involved. The differences in the studies may be due to several factors such as the use of different microarray method, methodological differences related to sample collection as well as the sample cohort size. Thus, the importance of validation in larger sample collections is underlined in order to separate meaningful findings from random occurrences. However, the genes described in the study presented here might indeed have a role in the pathogenesis of EOC and thus serve as interesting targets for future studies.

6.2 PARP in HGSC (II)

As mentioned in the Review of the Literature, the treatment of OC has been changed in recent years by the introduction of PARP inhibitors. PARPi is an effective targeted treatment with broadening indications and is transforming the care of HGSC patients.

Because PARPi have been proven to be especially efficient in platinum-sensitive, *BRCA*-mutated or otherwise HRD disease, the current study was designed to evaluate PARP protein in paraffin-embedded blocks and, more importantly, to measure the activity of PARP in OC tissue in correlation with platinum sensitivity. As a result, platinum sensitivity was associated with high PARP concentration in the entire study cohort and especially in the high-grade subgroup. With regard to survival, a high PARP concentration was associated with longer PFS and low PARP with an increased risk of recurrence. These are novel results not previously described in an EOC setting. To my knowledge, PARP activity has not been measured by pharmacodynamic assay in an OC setting from patient samples.

Platinum sensitivity serves as a marker for the BRCAness profile, and although *BRCA* mutation status was not available for the patients participating in this study, it can be argued that the patients with platinum sensitivity and favorable prognosis befit the BRCAness profile (Tan et al., 2008). This was considered when interpreting the results. The high-grade and platinum-sensitive subgroup association with PARP

activity is in line with this theory as well as the difference in progression-free survival between high PARP expression vs low PARP.

Interestingly, in this study, low PARP was associated with NACT, which may be the result of NACT affecting tumors by exploiting PARP. A possible explanation may also be NACT's effect on tumor biology, inducing alterations leading to diminished PARP activity. Both remain speculations, as there were no sequential samples available for NACT-treated patients.

In previous studies, the preferential use of repair mechanisms in the absence of HR often leads to DNA alterations, including deletions of genetic material (Lord and Ashworth, 2017), and through these alterations, NACT's negative effect on platinum response has been proposed (Sato and Itamochi, 2014). In an in vitro study, platinum resistance was common after NACT (Matsuo et al., 2010). In a retrospective study involving 341 patients, it was found that NACT may increase the risk of platinum resistance in stage IIIC and IV EOC (Luo et al., 2016). It must be stated, however, that prospective data on the matter are currently unavailable. Some biomarkers have been proposed: tumor necrosis factor α-induced protein 8 (TNFAIP8) may be involved via autophagy-related proteins (J. Wang et al., 2018). In another study, NACT and subsequent relapse and resistance were associated with the highest basal expression of HGF and c-Met, mediated by mir-193a-5p (Mariani et al., 2014), proposing that inhibitors of this pathway may improve the efficacy of NACT. The effect of NACT on tumor biology remains an interesting target for research.

In terms of ICH findings, PARP ICH and PARP activity did not correlate. PARP-1 has been assessed by IHC in an OC setting and has been found to be overexpressed, possibly enhancing angiogenesis by upregulating VEGF (W. Wei et al., 2016). On the other hand, a study from 2017 found no correlation between PARP IHC staining and the outcome (Hjortkjær et al., 2017). PARP expression measurement attempts have been made with different modalities, with a recent study incorporating radiotracer [18F]FluorThanatrace ([18F]FTT) as a marker of PARP expression in vitro to show the correlation of PARP-1 expression with response to PARPi treatment (Sander Effron et al., 2017). The result would need to be tested in patients' samples but is certainly promising. In clinical samples, PARP-1 overexpression was found in clear-cell and endometrioid carcinomas associating with early recurrence and worse OS (Barreta et al., 2018). These results are the opposite of the results presented in this study, although it must be noted that the focus of the current study was HGSC, which may in part explain the inconsistency. Due to poor reproducibility among other studies (no clear evidence related to

prognosis and effect on treatment), to date, PARP-1 expression is not routinely tested in the setting of OC.

The current study clarified that PARP IHC does not provide a clinical tool for assessment of PARP activity. PARP activity measured in fresh frozen tumor samples provides a more precise result in terms of PARP activity but is difficult and costly to obtain. The study described here did give new insight into interactions between platinum sensitivity, favorable prognosis and PARP activity.

6.3 Expression analysis in HGSC (III)

Acquired chemoresistance and its underlying mechanisms are discussed in detail in chapter 2.3.4 of this thesis. Keen efforts on the front of database research have been made to provide additional insight into chemoresistance in OC, in addition to basic mechanism and pathway-orientated research. Among others, dataset analysis has been deployed with the TCGA OC dataset, which was imputed with patient tumor responses to over 100 different drugs and of these, five drugs (ABT-888, BIBW2992, gefitinib, AZD6244 and lenalidomide) identified as potentially improving the clinical outcome of chemoresistant OC (Wang et al., 2017). These large-scale genomic database studies do enhance the understanding of basic molecular events in the tumorigenesis and chemoresistance of OC (Krzystyniak et al., 2016). However, this approach needs more elaborate testing and validation in vivo.

Although multiple mechanisms and approaches have been investigated, specific targeted treatment has not yet been introduced to clinical practice. There is also no prognostic marker available for chemoresistance testing that could be used in clinical work and patient assessment in order to avoid needless therapy.

To investigate some of the molecular mechanisms involved in OC chemoresistance, gene expression analysis of HGSC patient samples was assessed by microarray, qRT-PCR and Western blotting and OC cell line analyses. The sample cohorts were retrospective and specifically selected, taking into consideration also the results from study II involving PARP activity and platinum sensitivity. Specifically, the aim was to select groups representing on one hand a platinum-sensitive, high PARP activity, favorable prognosis group befitting the BRCAness profile and on the other hand, a low PARP activity, platinum-resistant, poor prognosis group. In addition, two other groups were formed – one with high PARP activity and platinum-resistant patients and the other with low PARP activity and

platinum sensitivity. These selections were made to maximize the data to be extracted from a relatively small sample size with special interest in chemoresistance.

As a result, *ROR2* was found to be downregulated in the microarray and validation analyses, and the results were further underlined by protein expression profiling. In addition, the levels of ROR2 (and STAT3, NF-kB and Wnt5a that are parts of the affected pathway) were increased in OC A2780cis cells in comparison to the parental cell line, A2780.

ROR2 is a receptor that is part of the noncanonical Wnt pathway (Katoh, 2005). Wnt signals either stabilize β-catenin in the nucleus (canonical/β-catenin-dependent) or elicit alternative routes of intracellular signaling independent of β-catenin stabilization (noncanonical/β-catenin-independent) (Roarty et al., 2017; Angers and Moon, 2009). Regulation of organized cellular movements, cell shape and the orientation of proper cell polarity are all transduced through noncanonical Wnt signals (van Amerongen, 2012). The Wnt pathway has been carefully studied and its parts identified (Klaus and Birchmeier, 2008). Wnt signaling and the role of ROR2 have been investigated in cancer and found to be present in several cancers as well as other diseases (Sumithra et al., 2016). More specifically, hereditary colorectal cancer, exudative vitreoretinopathy, bone diseases, and intellectual disability syndrome can be caused by germline mutations in Wnt signaling molecules (Katoh and Katoh, 2017). In OC, ROR2 has been shown to have a role in stromal activation and metastasis development through cell adhesion and migration mechanisms (Henry et al., 2017). ROR2 has also been demonstrated to play a role in chemoresistance by altering cell migration and invasion mechanisms (Henry et al., 2016). In the same study, it was proposed that ROR knockdown could sensitize OC cells to cisplatin and thus provide an option for treatment. This result, however, needs to be validated in other models to further investigate its attractiveness.

In the current study, *ROR2* gene expression was upregulated in platinum-resistant samples. The results are in concordance with previous results suggesting that this pathway could be involved in platinum resistance development. There is prior evidence that Wnt5a is involved in chemotherapy resistance in bladder and breast cancers (Cao et al., 2018; Zeng et al., 2016), although through differently mediated mechanisms that do not involve ROR receptors. There is also evidence that parts of the pathway, namely, STAT3, have a role in chemoresistance development in the OC setting. To be more specific, STAT3 is hyperactivated in OC spheroids, and targeting STAT3 in combination with paclitaxel reduces peritoneal seeding and prolongs survival in a murine model of OC. (Chen et al., 2017). The active role of the Wnt pathway in OC has thus been established, and a possibility for targeted

treatment has been underlined (Arend et al., 2013); our results offer further support to these prior investigations.

GREB1 was upregulated in the microarray data in the high PARP and platinum-sensitive patient group, and the result was validated by qRT-PCR. High expression of *GREB1* was associated with better survival (longer PFS).

GREB1 is a chromatin-bound ER coactivator and is essential for ER-mediated transcription by stabilizing interactions between ER and additional cofactors (Mohammed et al., 2013). *GREB1* was identified in a breast cancer cell line MCF7 as a hormone-responsive gene (Ghosh et al., 2000). In BC, the role of *GREB1* has been investigated (Cheng et al., 2018). GREB1 has been shown to be upregulated in breast cancer tissues, and the high expression level of GREB1 has been associated with the high grade and TNM stage of breast cancer (Ding et al., 2018).

In the OC setting, data have suggested that 86% of EOCs are ER-positive (Shen et al., 2017). Furthermore, estrogen can stimulate the proliferation of ER-positive OC cell lines (Langdon et al., 1994). GREB1 was expressed in 75–85% of EOC cells in OC tissue microarray analysis (Hodgkinson et al., 2018). It has been demonstrated in a transgenic mouse model of poorly differentiated EOC that exogenous E2 accelerates ovarian tumor initiation by causing precancerous lesions in the ovarian surface epithelium (Laviolette et al., 2010). Additionally, GREB1 has been identified in this setting, again in a mouse model, as a moderator in this reaction, and the knockdown of GREB1 decreased their proliferation rate in vitro and increased survival time in mice engrafted with the cells (Laviolette et al., 2014). In our study, that finding is supported in EOC tumor samples with regard to better survival and upregulation of *GREB1* in the platinum-sensitive and high PARP patient group that has a more favorable prognosis. Further investigations into hormone receptors and their regulation in association with GREB1 are warranted to uncover potential targets for therapeutics.

The aim of this study was to identify candidate genes and their respective molecular pathways involved in the pathogenesis and chemoresistance of HGSC. This is a subject extensively researched in the setting of OC, and although promising avenues of research have been opened up, a targeted treatment in clinical practice remains elusive to date. The results of this study investigating Wnt5a and ROR2 expression and their association with the development of platinum resistance do provide some additional insight into this field of research. Importantly, since ROR1 and ROR2 silencing restores the chemosensitivity of platinum-resistant OC cells (Henry et al., 2016), ROR might have the potential to influence and overturn that effect as a therapeutic agent. In addition, *GREB1* expression was for the first time

described in EOC tumor samples with an effect on survival. These findings provide possibilities for future investigations in terms of possible treatment options.

6.4 Limitations and advantages

There are some limitations to the research depicted in this thesis. In detail, the first publication unarguably has a small sample size. However, it must be pointed out that *BRCA*-mutation positive RRSO samples are difficult to collect. Although genetic testing for *BRCA 1/2* mutations has been available since 1996, only recently has more vigorous testing of *BRCA* mutations has been deployed in clinical work. Regardless, the mutations are quite rare, even if tested more often and that impacts significantly the speed and effectiveness of sample collection.

To further discuss the limitations, it must be noted that the *BRCA* status of the control samples is unverified, although it is very unlikely they harbor the mutation. In addition, tissue microdissection was not undertaken at the time of sample collection, and the results are thus limited in terms of cell collection precision. However, it must be noted that while physical cell separation techniques (i.e., microdissection) can enhance the sample collection and results derived, they are also costly, labor-intensive and might limit investigation of interactions between different cell types (Liebner et al., 2014). RNA extraction was performed from tissue containing both stromal and epithelial ovarian cells, doubtless impacting the results.

To discuss the techniques used, the microarray technique has been useful for assigning functions to previously annotated genes as well as for grouping genes into pathways (DeRisi et al., 1996). Concerns over the reliability of the technology have been raised, since contradictory results have been published using same RNA samples but different platforms (MAQC Consortium et al., 2006). This issue has been addressed in the ovarian cancer setting, and microarray quality control analysis has been performed. It demonstrated that 40% of the microarray files were false-negative and that 70% of the significantly expressed genes were correlated in separate analyses (Villegas-Ruiz et al., 2016). To address this, the Bioconductor package was used to ensure quality control (Kauffmann et al., 2009).

As an advantage, bioinformatics tools were used to group the large amount of data provided by microarray assay and thus the information retrieval possibilities were maximized. Furthermore, the patient cohort in the first study benefitted from harboring clearly identified *BRCA* mutations. In addition, it must be emphasized

that the study was designed as a hypothesis-generating exploration, serving as a means to provide candidates for further studies.

The second study in this thesis has a prospective sample cohort, which is an advantage. Ideally, for the second study, the *BRCA* or HRD status for the study patients would have been tested, and the lack of these data is a limitation. However, in view of the relatively substantial study cohort, the costs would have been unreasonable at the time the study was carried out.

In addition, the fresh frozen sample collection was done without intraoperative tissue verification. Intraoperative assessment by an experienced gynecological oncologist to assess the presence of tumor tissue in a specimen was used in this study, and in some cases, intraoperative frozen sections supported the assessments. However, in terms of NACT, there were no sequential samples collected, and the results in terms of NACT and PARP activity are thus limited.

In the third study, different techniques were deployed to the advantage of the results and their interpretation, namely, in addition to gene expression, protein expression was measured as well. OC cell line models were used to further underline the findings in the case of *ROR2*. The limitations include the lack of tumor tissue verification already discussed above. In addition, the carefully preselected study cohorts for microarray designed to provide information about chemoresistance also predispose to a bias. The PARP activity determined in study two was used to select the cohort in study three; however, the platinum sensitivity and more favorable BRCAness profile associated with high PARP activity may bear impact on the results the microarray yielded. It cannot thus be stressed enough that the results provided should be further validated in a larger sample cohort without specific preselection. Furthermore, the biological significance of the results in study III were not confirmed by employing mechanistic studies.

Additionally, for *GREB1* investigations, cell line work was not undertaken and remains a possible target for further investigations. However, *GREB1* expression in OC tumor samples has been demonstrated for the first time as a novel finding, and the importance is further underlined by the association with survival. A more extensive study design might provide additional evidence in the future.

7 FUTURE PROSPECTS

This research project provided many interesting prospects for further investigations.

In detail, the comparison of the RRSO samples in *BRCA* mutated and healthy controls provided several genes that may be involved in the development of HGSC. A more detailed investigation into the details and pathways of these mechanisms in a larger sample size are warranted for several of the genes, namely, *SIK1, KLF4* and *EGR3* as most interesting candidates based on their expression and data derived from previous literature. In addition to investigating the network surrounding the genes, OC cell line model work could provide insight into the genes' effects in vitro.

Regarding the PARP protein, the results provided here illustrated the difficulty of measuring PARP in paraffin-embedded blocks. Activity in tumor cells is more reliable for measuring PARP activity but acquiring the material suitable for that poses a more difficult task. In this study, platinum sensitivity was used as a surrogate marker for the BRCAness profile, which has previously been associated with a response to PARPi treatment (Lord and Ashworth, 2016). In this study, the platinum sensitivity was associated with high PARP activity, and it could be hypothesized that it would predict the response to PARPi. However, the testing of this hypothesis would require large, randomized studies involving PARPi treatment.

The results of the third study researching *ROR2* were encouraging. ROR receptors are part of the Wnt pathway; recently, Wnt pathway inhibitors have shown great promise in cancer treatment and are currently under evaluation as a potential therapeutic in several cancers (Ahmed et al., 2019; Zhu et al., 2018; Flanagan et al., 2017). A possible targeted therapy or a combination with chemotherapeutic agent might be a promising option in the setting of OC as well. Further detailing of the pathway and its affected components is certainly warranted.

GREB1 is also an interesting gene, possibly bearing an influence on the modulation of EOC via hormonal interactions, and thus further studies might be of interest.

8 SUMMARY AND CONCLUSIONS

This dissertation concentrated on the early pathogenesis of HGSC, as well as expression profiling at the gene and protein level, with special regard to treatment response. Specifically, it was revealed that:

1. In *BRCA1/2*-mutation-positive RRSO samples compared to healthy controls, there were interesting differentially expressed genes revealed by microarray analysis. From these, selected genes were validated by qRT-PCR, demonstrating comparable expression patterns between *BRCA1/2*-mutation-positive RRSO and HGSC samples. Thus, this study revealed potential candidate genes for future research on EOC pathogenesis and targeted therapy.

2. PARP expression in EOC tumor tissue was investigated by a PARP pharmacodynamic assay and PARP IHC staining. Interestingly, an association between high PARP activity and platinum sensitivity and longer PFS was observed, which is a novel finding. Furthermore, NACT seemed to be associated with low PARP activity. The IHC staining and pharmacodynamic assay PARP activity measurements did not associate. Based on the results, biologically significant PARP seems to be indicated by PARP pharmacodynamic assay rather than PARP IHC, which is, based on this study, not a clinically usable surrogate marker for PARP activity.

3. As a novel result, *ROR2* and *GREB1* were associated with treatment response in HGSC. Furthermore, the Wnt5a/ROR2 pathway is potentially actionable in the possible modulation of chemoresistance. A combination of *ROR* antagonists and chemotherapeutic agents may be an investigation-worthy option in the future.

9 REFERENCES

Abdullah, L.N., Chow, E.K.-H., 2013. Mechanisms of chemoresistance in cancer stem cells. Clin. Transl. Med. 2, 3.

Aghajanian, C., Blank, S.V., Goff, B.A., Judson, P.L., Teneriello, M.G., Husain, A., Sovak, M.A., Yi, J., Nycum, L.R., 2012. OCEANS: a randomized, double-blind, placebo-controlled phase III trial of chemotherapy with or without bevacizumab in patients with platinum-sensitive recurrent epithelial ovarian, primary peritoneal, or fallopian tube cancer. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 30, 2039–2045.

Ahmed, K., Koval, A., Xu, J., Bodmer, A., Katanaev, V.L., 2019. Towards the first targeted therapy for triple-negative breast cancer: Repositioning of clofazimine as a chemotherapy-compatible selective Wnt pathway inhibitor. Cancer Lett. 449, 45–55.

Aletti, G.D., Dowdy, S., Podratz, K.C., Cliby, W.A., 2006. Role of lymphadenectomy in the management of grossly apparent advanced stage epithelial ovarian cancer. Am. J. Obstet. Gynecol. 195, 1862–1868.

Andrews, L., Mutch, D.G., 2017. Hereditary Ovarian Cancer and Risk Reduction. Best Pract. Res. Clin. Obstet. Gynaecol. 41, 31–48.

Ang, C., Chan, K.K.L., Bryant, A., Naik, R., Dickinson, H.O., 2011. Ultra-radical (extensive) surgery versus standard surgery for the primary cytoreduction of advanced epithelial ovarian cancer. Cochrane Database Syst. Rev. CD007697.

Ang, J.E., Gourley, C., Powell, C.B., High, H., Shapira-Frommer, R., Castonguay, V., De Greve, J., Atkinson, T., Yap, T.A., Sandhu, S., Banerjee, S., Chen, L.-M., Friedlander, M.L., Kaufman, B., Oza, A.M., Matulonis, U., Barber, L.J., Kozarewa, I., Fenwick, K., Assiotis, I., Campbell, J., Chen, L., de Bono, J.S., Gore, M.E., Lord, C.J., Ashworth, A., Kaye, S.B., 2013. Efficacy of chemotherapy in BRCA1/2 mutation carrier ovarian cancer in the setting of PARP inhibitor resistance: a multi-institutional study. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 19, 5485–5493.

Angeles, M.A., Martínez-Gómez, C., Migliorelli, F., Voglimacci, M., Figurelli, J., Motton, S., Tanguy Le Gac, Y., Ferron, G., Martinez, A., 2018. Novel Surgical Strategies in the Treatment of Gynecological Malignancies. Curr. Treat. Options Oncol. 19, 73.

Angers, S., Moon, R.T., 2009. Proximal events in Wnt signal transduction. Nat. Rev. Mol. Cell Biol. 10, 468–477.

Archambault, V., Lépine, G., Kachaner, D., 2015. Understanding the Polo Kinase machine. Oncogene 34, 4799–4807.

Arend, R.C., Londoño-Joshi, A.I., Straughn, J.M., Buchsbaum, D.J., 2013. The Wnt/β-catenin pathway in ovarian cancer: a review. Gynecol. Oncol. 131, 772–779.

Armstrong, D.K., Bundy, B., Wenzel, L., Huang, H.Q., Baergen, R., Lele, S., Copeland, L.J., Walker, J.L., Burger, R.A., Gynecologic Oncology Group, 2006. Intraperitoneal cisplatin and paclitaxel in ovarian cancer. N. Engl. J. Med. 354, 34–43.

Balacescu, O., Balacescu, L., Tudoran, O., Todor, N., Rus, M., Buiga, R., Susman, S., Fetica, B., Pop, L., Maja, L., Visan, S., Ordeanu, C., Berindan-Neagoe, I., Nagy, V., 2014. Gene expression profiling reveals activation of the FA/BRCA pathway in advanced squamous cervical cancer with intrinsic resistance and therapy failure. BMC Cancer 14, 246.

Barber, L.J., Sandhu, S., Chen, L., Campbell, J., Kozarewa, I., Fenwick, K., Assiotis, I., Rodrigues, D.N., Reis Filho, J.S., Moreno, V., Mateo, J., Molife, L.R., De Bono, J., Kaye, S., Lord, C.J., Ashworth, A., 2013. Secondary mutations in BRCA2 associated with clinical resistance to a PARP inhibitor. J. Pathol. 229, 422–429.

Barreta, A., Sarian, L.O., Ferracini, A.C., Costa, L.B.E., Mazzola, P.G., de Angelo Andrade, L., Derchain, S., 2018. Immunohistochemistry expression of targeted therapies biomarkers in ovarian clear cell and endometrioid carcinomas (type I) and endometriosis. Hum. Pathol.

Bast, R.C., 2011. Molecular approaches to personalizing management of ovarian cancer. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 22 Suppl 8, viii5–viii15.

Bast, R.C., Hennessy, B., Mills, G.B., 2009. The biology of ovarian cancer: new opportunities for translation. Nat. Rev. Cancer 9, 415–428.

Bast, R.C., Xu, F.J., Yu, Y.H., Barnhill, S., Zhang, Z., Mills, G.B., 1998. CA 125: the past and

the future. Int. J. Biol. Markers 13, 179–187.

Bekes, I., Friedl, T.W.P., Köhler, T., Möbus, V., Janni, W., Wöckel, A., Wulff, C., 2016. Does VEGF facilitate local tumor growth and spread into the abdominal cavity by suppressing endothelial cell adhesion, thus increasing vascular peritoneal permeability followed by ascites production in ovarian cancer? Mol. Cancer 15, 13.

Bhattacharjee, S., Nandi, S., 2017. Synthetic lethality in DNA repair network: A novel avenue in targeted cancer therapy and combination therapeutics. IUBMB Life 69, 929–937.

Bian, K., Muppani, N.R., Elkhadragy, L., Wang, W., Zhang, C., Chen, T., Jung, S., Seternes, O.M., Long, W., 2016. ERK3 regulates TDP2-mediated DNA damage response and chemoresistance in lung cancer cells. Oncotarget 7, 6665–6675.

Binder, P.S., Prat, J., Mutch, D.G., 2015. Molecular staging of gynecological cancer: What is the future? Best Pract. Res. Clin. Obstet. Gynaecol. 29, 776–789.

Birmann, B.M., Barnard, M.E., Bertrand, K.A., Bao, Y., Crous-Bou, M., Wolpin, B.M., De Vivo, I., Tworoger, S.S., 2016. Nurses' Health Study Contributions on the Epidemiology of Less Common Cancers: Endometrial, Ovarian, Pancreatic, and Hematologic. Am. J. Public Health 106, 1608–1615.

Blok, L.J., Grossmann, M.E., Perry, J.E., Tindall, D.J., 1995. Characterization of an early growth response gene, which encodes a zinc finger transcription factor, potentially involved in cell cycle regulation. Mol. Endocrinol. Baltim. Md 9, 1610–1620.

Bolstad, B.M., Irizarry, R.A., Astrand, M., Speed, T.P., 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinforma. Oxf. Engl. 19, 185–193.

Bookman, M.A., Brady, M.F., McGuire, W.P., Harper, P.G., Alberts, D.S., Friedlander, M., Colombo, N., Fowler, J.M., Argenta, P.A., De Geest, K., Mutch, D.G., Burger, R.A., Swart, A.M., Trimble, E.L., Accario-Winslow, C., Roth, L.M., 2009. Evaluation of new platinum-based treatment regimens in advanced-stage ovarian cancer: a Phase III Trial of the Gynecologic Cancer Intergroup. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 27, 1419–1425.

Bottoni, P., Scatena, R., 2015. The Role of CA 125 as Tumor Marker: Biochemical and Clinical Aspects. Adv. Exp. Med. Biol. 867, 229–244.

Boussios, S., Moschetta, M., Zarkavelis, G., Papadaki, A., Kefas, A., Tatsi, K., 2017. Ovarian sex-cord stromal tumours and small cell tumours: Pathological, genetic and management aspects. Crit. Rev. Oncol. Hematol. 120, 43–51.

Bouwman, P., Jonkers, J., 2012. The effects of deregulated DNA damage signalling on cancer chemotherapy response and resistance. Nat. Rev. Cancer 12, 587–598.

Bristow, R.E., Tomacruz, R.S., Armstrong, D.K., Trimble, E.L., Montz, F.J., 2002. Survival effect of maximal cytoreductive surgery for advanced ovarian carcinoma during the platinum era: a meta-analysis. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 20, 1248–1259.

Burger, R.A., Brady, M.F., Bookman, M.A., Fleming, G.F., Monk, B.J., Huang, H., Mannel, R.S., Homesley, H.D., Fowler, J., Greer, B.E., Boente, M., Birrer, M.J., Liang, S.X., Gynecologic Oncology Group, 2011. Incorporation of bevacizumab in the primary treatment of ovarian cancer. N. Engl. J. Med. 365, 2473–2483.

Burghardt, E., Girardi, F., Lahousen, M., Tamussino, K., Stettner, H., 1991. Patterns of pelvic and paraaortic lymph node involvement in ovarian cancer. Gynecol. Oncol. 40, 103–106.

Buys, S.S., Partridge, E., Black, A., Johnson, C.C., Lamerato, L., Isaacs, C., Reding, D.J., Greenlee, R.T., Yokochi, L.A., Kessel, B., Crawford, E.D., Church, T.R., Andriole, G.L., Weissfeld, J.L., Fouad, M.N., Chia, D., O'Brien, B., Ragard, L.R., Clapp, J.D., Rathmell, J.M., Riley, T.L., Hartge, P., Pinsky, P.F., Zhu, C.S., Izmirlian, G., Kramer, B.S., Miller, A.B., Xu, J.-L., Prorok, P.C., Gohagan, J.K., Berg, C.D., PLCO Project Team, 2011. Effect of screening on ovarian cancer mortality: the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Randomized Controlled Trial. JAMA 305, 2295–2303.

Callahan, M.J., Crum, C.P., Medeiros, F., Kindelberger, D.W., Elvin, J.A., Garber, J.E., Feltmate, C.M., Berkowitz, R.S., Muto, M.G., 2007. Primary fallopian tube malignancies in BRCA-positive women undergoing surgery for ovarian cancer risk reduction. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 25, 3985–3990.

Cao, J., Wang, Qichao, Wu, G., Li, S., Wang, Qian, 2018. miR-129-5p inhibits gemcitabine resistance and promotes cell apoptosis of bladder cancer cells by targeting Wnt5a. Int. Urol. Nephrol. 50, 1811–1819.

Caruso, D., Papa, A., Tomao, S., Vici, P., Panici, P.B., Tomao, F., 2017. Niraparib in ovarian

cancer: results to date and clinical potential. Ther. Adv. Med. Oncol. 9, 579–588.

Castellarin, M., Milne, K., Zeng, T., Tse, K., Mayo, M., Zhao, Y., Webb, J.R., Watson, P.H., Nelson, B.H., Holt, R.A., 2013. Clonal evolution of high-grade serous ovarian carcinoma from primary to recurrent disease. J. Pathol. 229, 515–524.

Chan, J.K., Brady, M.F., Penson, R.T., Huang, H., Birrer, M.J., Walker, J.L., DiSilvestro, P.A., Rubin, S.C., Martin, L.P., Davidson, S.A., Huh, W.K., O'Malley, D.M., Boente, M.P., Michael, H., Monk, B.J., 2016. Weekly vs. Every-3-Week Paclitaxel and Carboplatin for Ovarian Cancer. N. Engl. J. Med. 374, 738–748.

Chang, S.-J., Hodeib, M., Chang, J., Bristow, R.E., 2013. Survival impact of complete cytoreduction to no gross residual disease for advanced-stage ovarian cancer: a meta-analysis. Gynecol. Oncol. 130, 493–498.

Chen, J.-L., Chen, F., Zhang, T.-T., Liu, N.-F., 2016. Suppression of SIK1 by miR-141 in human ovarian cancer cell lines and tissues. Int. J. Mol. Med. 37, 1601–1610.

Chen, M.-W., Yang, S.-T., Chien, M.-H., Hua, K.-T., Wu, C.-J., Hsiao, S.M., Lin, H., Hsiao, M., Su, J.-L., Wei, L.-H., 2017. The STAT3-miRNA-92-Wnt Signaling Pathway Regulates Spheroid Formation and Malignant Progression in Ovarian Cancer. Cancer Res. 77, 1955–1967.

Chen, Q., Zhu, L., Zheng, B., Wang, Jinliang, Song, X., Zheng, W., Wang, L., Yang, D., Wang, Jianbo, 2016. Effect of AQP9 Expression in Androgen-Independent Prostate Cancer Cell PC3. Int. J. Mol. Sci. 17.

Chen, X., Zhou, C., Yan, C., Ma, J., Zheng, W., 2015. Hyperosmotic stress induces cisplatin sensitivity in ovarian cancer cells by stimulating aquaporin-5 expression. Exp. Ther. Med. 10, 2055–2062.

Chen, Y., Bi, F., An, Y., Yang, Q., 2018. Coexpression network analysis identified Krüppel-like factor 6 (KLF6) association with chemosensitivity in ovarian cancer. J. Cell. Biochem.

Chen, Z., Wang, Y., Liu, W., Zhao, G., Lee, S., Balogh, A., Zou, Y., Guo, Y., Zhang, Z., Gu, W., Li, C., Tigyi, G., Yue, J., 2014. Doxycycline inducible Krüppel-like factor 4 lentiviral vector mediates mesenchymal to epithelial transition in ovarian cancer cells. PloS One 9, e105331.

Cheng, H., Liu, P., Wang, Z.C., Zou, L., Santiago, S., Garbitt, V., Gjoerup, O.V., Iglehart, J.D., Miron, A., Richardson, A.L., Hahn, W.C., Zhao, J.J., 2009. SIK1 couples LKB1 to p53-dependent anoikis and suppresses metastasis. Sci. Signal. 2, ra35.

Cheng, M., Michalski, S., Kommagani, R., 2018. Role for Growth Regulation by Estrogen in Breast Cancer 1 (GREB1) in Hormone-Dependent Cancers. Int. J. Mol. Sci. 19.

Chetry, M., Li, S., Liu, H., Hu, X., Zhu, X., 2018. Prognostic values of aquaporins mRNA expression in human ovarian cancer. Biosci. Rep. 38.

Chien, M.-H., Lee, W.-J., Yang, Y.-C., Li, Y.-L., Chen, B.-R., Cheng, T.-Y., Yang, P.-W., Wang, M.-Y., Jan, Y.-H., Lin, Y.-K., Lee, J.-M., Hsiao, M., Chen, J.-S., Hua, K.-T., 2017. KSRP suppresses cell invasion and metastasis through miR-23a-mediated EGR3 mRNA degradation in non-small cell lung cancer. Biochim. Biophys. Acta Gene Regul. Mech. 1860, 1013–1024.

Christie, E.L., Bowtell, D.D.L., 2017. Acquired chemotherapy resistance in ovarian cancer. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 28, viii13–viii15.

Ciccia, A., Elledge, S.J., 2010. The DNA damage response: making it safe to play with knives. Mol. Cell 40, 179–204.

Clamp AR, McNeish I, Dean A A, Galladro D, KimJW, O,Donnell D, et al, ICON8, 2017. a GCIG phase III randomised trial evaluating weekly dose-dense chemotherapy integration in first-line epithelial ovarian/fallopian tube/primary peritoneal carcinoma (EOC) treatment: results of primary progression-free survival (PFS) analysis. Ann Oncol, Ann Oncol. 2017, 627.

Clarke-Pearson, D.L., 2009. Clinical practice. Screening for ovarian cancer. N. Engl. J. Med. 361, 170–177.

Coburn, S.B., Bray, F., Sherman, M.E., Trabert, B., 2017. International patterns and trends in ovarian cancer incidence, overall and by histologic subtype. Int. J. Cancer 140, 2451–2460.

Coleman, R.L., Brady, M.F., Herzog, T.J., Sabbatini, P., Armstrong, D.K., Walker, J.L., Kim, B.-G., Fujiwara, K., Tewari, K.S., O'Malley, D.M., Davidson, S.A., Rubin, S.C., DiSilvestro, P., Basen-Engquist, K., Huang, H., Chan, J.K., Spirtos, N.M., Ashfaq, R., Mannel, R.S., 2017a. Bevacizumab and paclitaxel-carboplatin chemotherapy and secondary cytoreduction

in recurrent, platinum-sensitive ovarian cancer (NRG Oncology/Gynecologic Oncology Group study GOG-0213): a multicentre, open-label, randomised, phase 3 trial. Lancet Oncol. 18, 779–791.

Coleman, R.L., Oza, A.M., Lorusso, D., Aghajanian, C., Oaknin, A., Dean, A., Colombo, N., Weberpals, J.I., Clamp, A., Scambia, G., Leary, A., Holloway, R.W., Gancedo, M.A., Fong, P.C., Goh, J.C., O'Malley, D.M., Armstrong, D.K., Garcia-Donas, J., Swisher, E.M., Floquet, A., Konecny, G.E., McNeish, I.A., Scott, C.L., Cameron, T., Maloney, L., Isaacson, J., Goble, S., Grace, C., Harding, T.C., Raponi, M., Sun, J., Lin, K.K., Giordano, H., Ledermann, J.A., ARIEL3 investigators, 2017b. Rucaparib maintenance treatment for recurrent ovarian carcinoma after response to platinum therapy (ARIEL3): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Lond. Engl. 390, 1949–1961.

Cook, L.S., Leung, A.C.Y., Swenerton, K., Gallagher, R.P., Magliocco, A., Steed, H., Koebel, M., Nation, J., Eshragh, S., Brooks-Wilson, A., Le, N.D., 2016. Adult lifetime alcohol consumption and invasive epithelial ovarian cancer risk in a population-based case-control study. Gynecol. Oncol. 140, 277–284.

Cordeiro Vidal, G., Babin, G., Querleu, D., Guyon, F., 2017. Primary debulking surgery of the upper abdomen and the diaphragm, with a plasma device surgery system, for advanced ovarian cancer. Gynecol. https://doi.org/10.1016/j.ygyno.2016.10.030

Cornelison, R., Llaneza, D.C., Landen, C.N., 2017. Emerging Therapeutics to Overcome Chemoresistance in Epithelial Ovarian Cancer: A Mini-Review. Int. J. Mol. Sci. 18.

Cowan, R.A., O'Cearbhaill, R.E., Zivanovic, O., Chi, D.S., 2017. Current status and future prospects of hyperthermic intraoperative intraperitoneal chemotherapy (HIPEC) clinical trials in ovarian cancer. Int. J. Hyperth. Off. J. Eur. Soc. Hyperthermic Oncol. North Am. Hyperth. Group 33, 548–553.

Crum, C.P., 2009. Intercepting pelvic cancer in the distal fallopian tube: theories and realities. Mol. Oncol. 3, 165–170.

Crum, C.P., Herfs, M., Ning, G., Bijron, J.G., Howitt, B.E., Jimenez, C.A., Hanamornroongruang, S., McKeon, F.D., Xian, W., 2013. Through the glass darkly: intraepithelial neoplasia, top-down differentiation, and the road to ovarian cancer. J. Pathol. 231, 402–412.

Davar, D., Beumer, J.H., Hamieh, L., Tawbi, H., 2012. Role of PARP inhibitors in cancer biology and therapy. Curr. Med. Chem. 19, 3907–3921.

David W Chan, M.X.L., YS Ngan, H., 2012. Mechanisms of Chemoresistance in Human Ovarian Cancer at a Glance. Gynecol. Obstet. 02.

Dembo, A.J., 1984. Spontaneous mutation to chemotherapy resistance: implications of the Goldie-Coldman model for the management of ovarian cancer. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 2, 1311–1316.

Deng, C.X., Scott, F., 2000. Role of the tumor suppressor gene Brca1 in genetic stability and mammary gland tumor formation. Oncogene 19, 1059–1064.

DeRisi, J., Penland, L., Brown, P.O., Bittner, M.L., Meltzer, P.S., Ray, M., Chen, Y., Su, Y.A., Trent, J.M., 1996. Use of a cDNA microarray to analyse gene expression patterns in human cancer. Nat. Genet. 14, 457–460.

Di Saia, P.J., Creasman, W.T., Mannell, R.S., McMeekin, S., Mutch, D.G., 2018. Clinical gynecologic oncology.

Ding, M., Liu, Yuhan, Li, J., Yao, L., Liao, X., Xie, H., Yang, K., Zhou, Q., Liu, Yuchen, Huang, W., Cai, Z., 2018. Oestrogen promotes tumorigenesis of bladder cancer by inducing the enhancer RNA-eGREB1. J. Cell. Mol. Med. 22, 5919–5927.

Dixon, S.C., Nagle, C.M., Thrift, A.P., Pharoah, P.D., Pearce, C.L., Zheng, W., Painter, J.N., AOCS Group & Australian Cancer Study (Ovarian Cancer), Chenevix-Trench, G., Fasching, P.A., Beckmann, M.W., Lambrechts, D., Vergote, I., Lambrechts, S., Van Nieuwenhuysen, E., Rossing, M.A., Doherty, J.A., Wicklund, K.G., Chang-Claude, J., Rudolph, A., Moysich, K.B., Odunsi, K., Goodman, M.T., Wilkens, L.R., Thompson, P.J., Shvetsov, Y.B., Dörk, T., Park-Simon, T.-W., Hillemanns, P., Bogdanova, N., Butzow, R., Nevanlinna, H., Pelttari, L.M., Leminen, A., Modugno, F., Ness, R.B., Edwards, R.P., Kelley, J.L., Heitz, F., Karlan, B.Y., Kjær, S.K., Høgdall, E., Jensen, A., Goode, E.L., Fridley, B.L., Cunningham, J.M., Winham, S.J., Giles, G.G., Bruinsma, F., Milne, R.L., Southey, M.C., Hildebrandt, M.A.T., Wu, X., Lu, K.H., Liang, D., Levine, D.A., Bisogna, M., Schildkraut, J.M., Berchuck, A., Cramer, D.W., Terry, K.L., Bandera, E.V., Olson, S.H., Salvesen, H.B., Thomsen, L.C., Kopperud, R.K., Bjorge, L., Kiemeney, L.A., Massuger, L.F.A.G., Pejovic, T., Cook, L.S., Le, N.D., Swenerton, K.D., Brooks-Wilson, A., Kelemen, L.E., Lubiński, J., Huzarski, T., Gronwald, J., Menkiszak, J., Wentzensen, N., Brinton, L., Yang, H., Lissowska, J., Høgdall,

C.K., Lundvall, L., Song, H., Tyrer, J.P., Campbell, I., Eccles, D., Paul, J., Glasspool, R., Siddiqui, N., Whittemore, A.S., Sieh, W., McGuire, V., Rothstein, J.H., Narod, S.A., Phelan, C., Risch, H.A., McLaughlin, J.R., Anton-Culver, H., Ziogas, A., Menon, U., Gayther, S.A., Ramus, S.J., Gentry-Maharaj, A., Wu, A.H., Pike, M.C., Tseng, C.-C., Kupryjanczyk, J., Dansonka-Mieszkowska, A., Budzilowska, A., Spiewankiewicz, B., Webb, P.M., Ovarian Cancer Association Consortium, 2016. Adult body mass index and risk of ovarian cancer by subtype: a Mendelian randomization study. Int. J. Epidemiol. 45, 884–895.

Doubeni, C.A., Doubeni, A.R., Myers, A.E., 2016. Diagnosis and Management of Ovarian Cancer. Am. Fam. Physician 93, 937–944.

Dréan, A., Lord, C.J., Ashworth, A., 2016. PARP inhibitor combination therapy. Crit. Rev. Oncol. Hematol. 108, 73–85.

du Bois, A., Reuss, A., Pujade-Lauraine, E., Harter, P., Ray-Coquard, I., Pfisterer, J., 2009a. Role of surgical outcome as prognostic factor in advanced epithelial ovarian cancer: a combined exploratory analysis of 3 prospectively randomized phase 3 multicenter trials: by the Arbeitsgemeinschaft Gynaekologische Onkologie Studiengruppe Ovarialkarzinom (AGO-OVAR) and the Groupe d'Investigateurs Nationaux Pour les Etudes des Cancers de l'Ovaire (GINECO). Cancer 115, 1234–1244.

du Bois, A., Reuss, A., Pujade-Lauraine, E., Harter, P., Ray-Coquard, I., Pfisterer, J., 2009b. Role of surgical outcome as prognostic factor in advanced epithelial ovarian cancer: a combined exploratory analysis of 3 prospectively randomized phase 3 multicenter trials: by the Arbeitsgemeinschaft Gynaekologische Onkologie Studiengruppe Ovarialkarzinom (AGO-OVAR) and the Groupe d'Investigateurs Nationaux Pour les Etudes des Cancers de l'Ovaire (GINECO). Cancer 115, 1234–1244.

Dubeau, L., 2008. The cell of origin of ovarian epithelial tumours. Lancet Oncol. 9, 1191–1197.

Ducie, J., Dao, F., Considine, M., Olvera, N., Shaw, P.A., Kurman, R.J., Shih, I.-M., Soslow, R.A., Cope, L., Levine, D.A., 2017. Molecular analysis of high-grade serous ovarian carcinoma with and without associated serous tubal intra-epithelial carcinoma. Nat. Commun. 8, 990.

Eckert, M.A., Pan, S., Hernandez, K.M., Loth, R.M., Andrade, J., Volchenboum, S.L., Faber, P., Montag, A., Lastra, R., Peter, M.E., Yamada, S.D., Lengyel, E., 2016. Genomics of

Ovarian Cancer Progression Reveals Diverse Metastatic Trajectories Including Intraepithelial Metastasis to the Fallopian Tube. Cancer Discov. 6, 1342–1351.

Edmonds, D.K., Dewhurst, J. (Eds.), 2007. Dewhurst's textbook of obstetrics & gynaecology, 7th ed. ed. Blackwell Pub, Malden, Mass.

Eisenkop, S.M., Friedman, R.L., Wang, H.J., 1998. Complete cytoreductive surgery is feasible and maximizes survival in patients with advanced epithelial ovarian cancer: a prospective study. Gynecol. Oncol. 69, 103–108.

Eisenkop, S.M., Spirtos, N.M., 2001. Procedures required to accomplish complete cytoreduction of ovarian cancer: is there a correlation with "biological aggressiveness" and survival? Gynecol. Oncol. 82, 435–441.

Ethier, J.-L., Desautels, D.N., Templeton, A.J., Oza, A., Amir, E., Lheureux, S., 2017. Is the neutrophil-to-lymphocyte ratio prognostic of survival outcomes in gynecologic cancers? A systematic review and meta-analysis. Gynecol. Oncol. 145, 584–594.

Evans, P.M., Liu, C., 2008. Roles of Krüpel-like factor 4 in normal homeostasis, cancer and stem cells. Acta Biochim. Biophys. Sin. 40, 554–564.

Falconer, H., Yin, L., Grönberg, H., Altman, D., 2015. Ovarian cancer risk after salpingectomy: a nationwide population-based study. J. Natl. Cancer Inst. 107.

Farmer, H., McCabe, N., Lord, C.J., Tutt, A.N.J., Johnson, D.A., Richardson, T.B., Santarosa, M., Dillon, K.J., Hickson, I., Knights, C., Martin, N.M.B., Jackson, S.P., Smith, G.C.M., Ashworth, A., 2005. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 434, 917–921.

Fathalla, M.F., 1971. Incessant ovulation--a factor in ovarian neoplasia? Lancet Lond. Engl. 2, 163.

Finch, A., Shaw, P., Rosen, B., Murphy, J., Narod, S.A., Colgan, T.J., 2006. Clinical and pathologic findings of prophylactic salpingo-oophorectomies in 159 BRCA1 and BRCA2 carriers. Gynecol. Oncol. 100, 58–64.

Flanagan, D.J., Vincan, E., Phesse, T.J., 2017. Winding back Wnt signalling: potential therapeutic targets for treating gastric cancers. Br. J. Pharmacol. 174, 4666–4683.

Ford, D., Easton, D.F., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D.T., Weber, B., Lenoir, G., Chang-Claude, J., Sobol, H., Teare, M.D., Struewing, J., Arason, A., Scherneck, S., Peto, J., Rebbeck, T.R., Tonin, P., Neuhausen, S., Barkardottir, R., Eyfjord, J., Lynch, H., Ponder, B.A., Gayther, S.A., Zelada-Hedman, M., 1998. Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. Am. J. Hum. Genet. 62, 676–689.

Fortner, R.T., Ose, J., Merritt, M.A., Schock, H., Tjønneland, A., Hansen, L., Overvad, K., Dossus, L., Clavel-Chapelon, F., Baglietto, L., Boeing, H., Trichopoulou, A., Benetou, V., Lagiou, P., Agnoli, C., Mattiello, A., Masala, G., Tumino, R., Sacerdote, C., Bueno-de-Mesquita, H.B.A., Onland-Moret, N.C., Peeters, P.H., Weiderpass, E., Torhild Gram, I., Duell, E.J., Larrañaga, N., Ardanaz, E., Sánchez, M.-J., Chirlaque, M.-D., Brändstedt, J., Idahl, A., Lundin, E., Khaw, K.-T., Wareham, N., Travis, R.C., Rinaldi, S., Romieu, I., Gunter, M.J., Riboli, E., Kaaks, R., 2015. Reproductive and hormone-related risk factors for epithelial ovarian cancer by histologic pathways, invasiveness and histologic subtypes: Results from the EPIC cohort. Int. J. Cancer 137, 1196–1208.

Gadducci, A., Guarneri, V., Peccatori, F.A., Ronzino, G., Scandurra, G., Zamagni, C., Zola, P., Salutari, V., 2019. Current strategies for the targeted treatment of high-grade serous epithelial ovarian cancer and relevance of BRCA mutational status. J. Ovarian Res. 12, 9.

Gao, Z., Deng, W., Zhu, F., 2019. Reference gene selection for quantitative gene expression analysis in black soldier fly (Hermetia illucens). PloS One 14, e0221420.

Geacintov, N.E., Broyde, S., 2017. Repair-Resistant DNA Lesions. Chem. Res. Toxicol. 30, 1517–1548.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y.H., Zhang, J., 2004a. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y.H., Zhang, J., 2004b. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80.

George, S.H., Greenaway, J., Milea, A., Clary, V., Shaw, S., Sharma, M., Virtanen, C., Shaw, P.A., 2011. Identification of abrogated pathways in fallopian tube epithelium from BRCA1 mutation carriers. J. Pathol. 225, 106–117.

Ghosh, M.G., Thompson, D.A., Weigel, R.J., 2000. PDZK1 and GREB1 are estrogen-regulated genes expressed in hormone-responsive breast cancer. Cancer Res. 60, 6367–6375.

Gloss, B.S., Samimi, G., 2014. Epigenetic biomarkers in epithelial ovarian cancer. Cancer Lett. 342, 257–263.

Goff, B.A., Agnew, K., Neradilek, M.B., Gray, H.J., Liao, J.B., Urban, R.R., 2017. Combining a symptom index, CA125 and HE4 (triple screen) to detect ovarian cancer in women with a pelvic mass. Gynecol. Oncol. 147, 291–295.

Gómez-Herreros, F., Zagnoli-Vieira, G., Ntai, I., Martínez-Macías, M.I., Anderson, R.M., Herrero-Ruíz, A., Caldecott, K.W., 2017. TDP2 suppresses chromosomal translocations induced by DNA topoisomerase II during gene transcription. Nat. Commun. 8, 233.

Gottesman, M.M., 2002. Mechanisms of cancer drug resistance. Annu. Rev. Med. 53, 615–627.

Greimel, E.R., Bjelic-Radisic, V., Pfisterer, J., Hilpert, F., Daghofer, F., du Bois, A., Arbeitsgemeinschaft Gynaekologische Onkologie Ovarian Cancer Study Group, 2006. Randomized study of the Arbeitsgemeinschaft Gynaekologische Onkologie Ovarian Cancer Study Group comparing quality of life in patients with ovarian cancer treated with cisplatin/paclitaxel versus carboplatin/paclitaxel. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 24, 579–586.

Gulhan, D.C., Lee, J.J.-K., Melloni, G.E.M., Cortés-Ciriano, I., Park, P.J., 2019. Detecting the mutational signature of homologous recombination deficiency in clinical samples. Nat. Genet. 51, 912–919.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646–674.

Hanahan, D., Weinberg, R.A., 2000. The hallmarks of cancer. Cell 100, 57–70.

Hankinson, S.E., Hunter, D.J., Colditz, G.A., Willett, W.C., Stampfer, M.J., Rosner, B.,

Hennekens, C.H., Speizer, F.E., 1993. Tubal ligation, hysterectomy, and risk of ovarian cancer. A prospective study. JAMA 270, 2813–2818.

Harter, P., Sehouli, J., Lorusso, D., Reuss, A., Vergote, I., Marth, C., Kim, J.-W., Raspagliesi, F., Lampe, B., Aletti, G., Meier, W., Cibula, D., Mustea, A., Mahner, S., Runnebaum, I.B., Schmalfeldt, B., Burges, A., Kimmig, R., Scambia, G., Greggi, S., Hilpert, F., Hasenburg, A., Hillemanns, P., Giorda, G., von Leffern, I., Schade-Brittinger, C., Wagner, U., du Bois, A., 2019. A Randomized Trial of Lymphadenectomy in Patients with Advanced Ovarian Neoplasms. N. Engl. J. Med. 380, 822–832.

Hartmann, L.C., Lindor, N.M., 2016. The Role of Risk-Reducing Surgery in Hereditary Breast and Ovarian Cancer. N. Engl. J. Med. 374, 454–468.

Hauptmann, S., Friedrich, K., Redline, R., Avril, S., 2017. Ovarian borderline tumors in the 2014 WHO classification: evolving concepts and diagnostic criteria. Virchows Arch. Int. J. Pathol. 470, 125–142.

Hayat, M., 1984. [Adjuvant chemotherapy of cancer: definition, prognosis, statistical aspects]. Bull. Cancer (Paris) 71, 281–286.

Heidemann, L.N., Hartwell, D., Heidemann, C.H., Jochumsen, K.M., 2014. The relation between endometriosis and ovarian cancer - a review. Acta Obstet. Gynecol. Scand. 93, 20–31.

Hengel, S.R., Spies, M.A., Spies, M., 2017. Small-Molecule Inhibitors Targeting DNA Repair and DNA Repair Deficiency in Research and Cancer Therapy. Cell Chem. Biol. 24, 1101–1119.

Henry, C., Hacker, N., Ford, C., 2017. Silencing ROR1 and ROR2 inhibits invasion and adhesion in an organotypic model of ovarian cancer metastasis. Oncotarget 8, 112727–112738.

Henry, C E, Llamosas, E., Djordjevic, A., Hacker, N.F., Ford, C.E., 2016. Migration and invasion is inhibited by silencing ROR1 and ROR2 in chemoresistant ovarian cancer. Oncogenesis 5, e226–e226.

Henry, C. E., Llamosas, E., Djordjevic, A., Hacker, N.F., Ford, C.E., 2016. Migration and invasion is inhibited by silencing ROR1 and ROR2 in chemoresistant ovarian cancer.

Oncogenesis 5, e226.

Hjortkjær, M., Waldstrøm, M., Jakobsen, A., Kanstrup, H., Søgaard-Andersen, E., Dahl Steffensen, K., 2017. The Prognostic Value of BRCA1 and PARP Expression in Epithelial Ovarian Carcinoma: Immunohistochemical Detection. Int. J. Gynecol. Pathol. Off. J. Int. Soc. Gynecol. Pathol. 36, 180–189.

Hodgkinson, K., Forrest, L.A., Vuong, N., Garson, K., Djordjevic, B., Vanderhyden, B.C., 2018. GREB1 is an estrogen receptor-regulated tumour promoter that is frequently expressed in ovarian cancer. Oncogene 37, 5873–5886.

Hong, B., Zhang, J., Yang, W., 2018. Activation of the LKB1-SIK1 signaling pathway inhibits the TGF-β-mediated epithelial-mesenchymal transition and apoptosis resistance of ovarian carcinoma cells. Mol. Med. Rep. 17, 2837–2844.

Hsieh, M.-H., Chen, Yi-Ting, Chen, You-Tzung, Lee, Y.-H., Lu, J., Chien, C.-L., Chen, H.-F., Ho, H.-N., Yu, C.-J., Wang, Z.-Q., Teng, S.-C., 2017. PARP1 controls KLF4-mediated telomerase expression in stem cells and cancer cells. Nucleic Acids Res. 45, 10492–10503.

https://syoparekisteri.fi/tilastot/, n.d.

http://www.tenk.fi/fi/tenkin-ohjeistot, n.d.

Huang, H., Li, Y.J., Lan, C.Y., Huang, Q.D., Feng, Y.L., Huang, Y.W., Liu, J.H., 2013. Clinical significance of ascites in epithelial ovarian cancer. Neoplasma 60, 546–552.

Ibrahim, M., Yadav, S., Ogunleye, F., Zakalik, D., 2018. Male BRCA mutation carriers: clinical characteristics and cancer spectrum. BMC Cancer 18, 179.

Inagaki-Ohara, K., Kondo, T., Ito, M., Yoshimura, A., 2013. SOCS, inflammation, and cancer. JAK-STAT 2, e24053.

Iversen, L., Sivasubramaniam, S., Lee, A.J., Fielding, S., Hannaford, P.C., 2017. Lifetime cancer risk and combined oral contraceptives: the Royal College of General Practitioners' Oral Contraception Study. Am. J. Obstet. Gynecol. 216, 580.e1-580.e9.

Jackson, S.P., Bartek, J., 2009. The DNA-damage response in human biology and disease. Nature 461, 1071–1078.

Jacobs, I., Oram, D., Fairbanks, J., Turner, J., Frost, C., Grudzinskas, J.G., 1990. A risk of malignancy index incorporating CA 125, ultrasound and menopausal status for the accurate preoperative diagnosis of ovarian cancer. Br. J. Obstet. Gynaecol. 97, 922–929.

Jacobs, I.J., Menon, U., Ryan, A., Gentry-Maharaj, A., Burnell, M., Kalsi, J.K., Amso, N.N., Apostolidou, S., Benjamin, E., Cruickshank, D., Crump, D.N., Davies, S.K., Dawnay, A., Dobbs, S., Fletcher, G., Ford, J., Godfrey, K., Gunu, R., Habib, M., Hallett, R., Herod, J., Jenkins, H., Karpinskyj, C., Leeson, S., Lewis, S.J., Liston, W.R., Lopes, A., Mould, T., Murdoch, J., Oram, D., Rabideau, D.J., Reynolds, K., Scott, I., Seif, M.W., Sharma, A., Singh, N., Taylor, J., Warburton, F., Widschwendter, M., Williamson, K., Woolas, R., Fallowfield, L., McGuire, A.J., Campbell, S., Parmar, M., Skates, S.J., 2016. Ovarian cancer screening and mortality in the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS): a randomised controlled trial. Lancet Lond. Engl. 387, 945–956.

Januchowski, R., Wojtowicz, K., Sujka-Kordowska, P., Andrzejewska, M., Zabel, M., 2013. MDR gene expression analysis of six drug-resistant ovarian cancer cell lines. BioMed Res. Int. 2013, 241763.

Jarboe, E., Folkins, A., Nucci, M.R., Kindelberger, D., Drapkin, R., Miron, A., Lee, Y., Crum, C.P., 2008. Serous carcinogenesis in the fallopian tube: a descriptive classification. Int. J. Gynecol. Pathol. Off. J. Int. Soc. Gynecol. Pathol. 27, 1–9.

Jardin, F., Callanan, M., Penther, D., Ruminy, P., Troussard, X., Kerckaert, J.P., Figeac, M., Parmentier, F., Rainville, V., Vaida, I., Bertrand, P., Duval, A.B., Picquenot, J.M., Chaperot, L., Marolleau, J.P., Plumas, J., Tilly, H., Bastard, C., 2009. Recurrent genomic aberrations combined with deletions of various tumour suppressor genes may deregulate the G1/S transition in CD4+CD56+ haematodermic neoplasms and contribute to the aggressiveness of the disease. Leukemia 23, 698–707.

Javadi, S., Ganeshan, D.M., Qayyum, A., Iyer, R.B., Bhosale, P., 2016. Ovarian Cancer, the Revised FIGO Staging System, and the Role of Imaging. AJR Am. J. Roentgenol. 206, 1351–1360.

Jiang, J., Chan, Y.-S., Loh, Y.-H., Cai, J., Tong, G.-Q., Lim, C.-A., Robson, P., Zhong, S., Ng, H.-H., 2008. A core Klf circuitry regulates self-renewal of embryonic stem cells. Nat. Cell Biol. 10, 353–360.

Jiang, X., Li, X., Li, W., Bai, H., Zhang, Z., 2019. PARP inhibitors in ovarian cancer:

Sensitivity prediction and resistance mechanisms. J. Cell. Mol. Med.

Kaku, T., Ogawa, S., Kawano, Y., Ohishi, Y., Kobayashi, H., Hirakawa, T., Nakano, H., 2003. Histological classification of ovarian cancer. Med. Electron Microsc. Off. J. Clin. Electron Microsc. Soc. Jpn. 36, 9–17.

Kaldawy, A., Segev, Y., Lavie, O., Auslender, R., Sopik, V., Narod, S.A., 2016. Low-grade serous ovarian cancer: A review. Gynecol. Oncol. 143, 433–438.

Karnezis, A.N., Cho, K.R., Gilks, C.B., Pearce, C.L., Huntsman, D.G., 2017. The disparate origins of ovarian cancers: pathogenesis and prevention strategies. Nat. Rev. Cancer 17, 65–74.

Katoh, M., 2005. WNT/PCP signaling pathway and human cancer (review). Oncol. Rep. 14, 1583–1588.

Katoh, Masuko, Katoh, Masaru, 2017. Molecular genetics and targeted therapy of WNT-related human diseases (Review). Int. J. Mol. Med. 40, 587–606.

Katsumata, N., Yasuda, M., Takahashi, F., Isonishi, S., Jobo, T., Aoki, D., Tsuda, H., Sugiyama, T., Kodama, S., Kimura, E., Ochiai, K., Noda, K., Japanese Gynecologic Oncology Group, 2009. Dose-dense paclitaxel once a week in combination with carboplatin every 3 weeks for advanced ovarian cancer: a phase 3, open-label, randomised controlled trial. Lancet Lond. Engl. 374, 1331–1338.

Kauffmann, A., Gentleman, R., Huber, W., 2009. arrayQualityMetrics--a bioconductor package for quality assessment of microarray data. Bioinforma. Oxf. Engl. 25, 415–416.

Kehoe, S., Hook, J., Nankivell, M., Jayson, G.C., Kitchener, H., Lopes, T., Luesley, D., Perren, T., Bannoo, S., Mascarenhas, M., Dobbs, S., Essapen, S., Twigg, J., Herod, J., McCluggage, G., Parmar, M., Swart, A.-M., 2015. Primary chemotherapy versus primary surgery for newly diagnosed advanced ovarian cancer (CHORUS): an open-label, randomised, controlled, non-inferiority trial. Lancet Lond. Engl. 386, 249–257.

Khiewvan, B., Torigian, D.A., Emamzadehfard, S., Paydary, K., Salavati, A., Houshmand, S., Werner, T.J., Alavi, A., 2017. An update on the role of PET/CT and PET/MRI in ovarian cancer. Eur. J. Nucl. Med. Mol. Imaging 44, 1079–1091.

Kim, J., Park, E.Y., Kim, O., Schilder, J.M., Coffey, D.M., Cho, C.-H., Bast, R.C., 2018. Cell Origins of High-Grade Serous Ovarian Cancer. Cancers 10.

Kim, M.Y., Zhang, T., Kraus, W.L., 2005. Poly(ADP-ribosyl)ation by PARP-1: "PAR-laying" NAD+ into a nuclear signal. Genes Dev. 19, 1951–1967.

Kisielewski, R., Tołwińska, A., Mazurek, A., Laudański, P., 2013. Inflammation and ovarian cancer--current views. Ginekol. Pol. 84, 293–297.

Klaus, A., Birchmeier, W., 2008. Wnt signalling and its impact on development and cancer. Nat. Rev. Cancer 8, 387–398.

Köbel, M., Bak, J., Bertelsen, B.I., Carpen, O., Grove, A., Hansen, E.S., Levin Jakobsen, A.-M., Lidang, M., Måsbäck, A., Tolf, A., Gilks, C.B., Carlson, J.W., 2014. Ovarian carcinoma histotype determination is highly reproducible, and is improved through the use of immunohistochemistry. Histopathology 64, 1004–1013.

Komiyama, S., Kugimiya, T., Kubushiro, K., 2018. Safety and efficacy of neoadjuvant chemotherapy containing bevacizumab and interval debulking surgery for advanced epithelial ovarian cancer: A feasibility study. J. Surg. Oncol. 118, 687–693.

Konstantinopoulos, P.A., Ceccaldi, R., Shapiro, G.I., D'Andrea, A.D., 2015. Homologous Recombination Deficiency: Exploiting the Fundamental Vulnerability of Ovarian Cancer. Cancer Discov. 5, 1137–1154.

Konstantinopoulos, P.A., Spentzos, D., Karlan, B.Y., Taniguchi, T., Fountzilas, E., Francoeur, N., Levine, D.A., Cannistra, S.A., 2010. Gene expression profile of BRCAness that correlates with responsiveness to chemotherapy and with outcome in patients with epithelial ovarian cancer. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 28, 3555–3561.

Krzystyniak, J., Ceppi, L., Dizon, D.S., Birrer, M.J., 2016. Epithelial ovarian cancer: the molecular genetics of epithelial ovarian cancer. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 27 Suppl 1, i4–i10.

Kuchenbaecker, K.B., Hopper, J.L., Barnes, D.R., Phillips, K.-A., Mooij, T.M., Roos-Blom, M.-J., Jervis, S., van Leeuwen, F.E., Milne, R.L., Andrieu, N., Goldgar, D.E., Terry, M.B., Rookus, M.A., Easton, D.F., Antoniou, A.C., BRCA1 and BRCA2 Cohort Consortium, McGuffog, L., Evans, D.G., Barrowdale, D., Frost, D., Adlard, J., Ong, K.-R., Izatt, L.,

Tischkowitz, M., Eeles, R., Davidson, R., Hodgson, S., Ellis, S., Nogues, C., Lasset, C., Stoppa-Lyonnet, D., Fricker, J.-P., Faivre, L., Berthet, P., Hooning, M.J., van der Kolk, L.E., Kets, C.M., Adank, M.A., John, E.M., Chung, W.K., Andrulis, I.L., Southey, M., Daly, M.B., Buys, S.S., Osorio, A., Engel, C., Kast, K., Schmutzler, R.K., Caldes, T., Jakubowska, A., Simard, J., Friedlander, M.L., McLachlan, S.-A., Machackova, E., Foretova, L., Tan, Y.Y., Singer, C.F., Olah, E., Gerdes, A.-M., Arver, B., Olsson, H., 2017. Risks of Breast, Ovarian, and Contralateral Breast Cancer for BRCA1 and BRCA2 Mutation Carriers. JAMA 317, 2402–2416.

Kurman, R.J., Shih, I.-M., 2016. The Dualistic Model of Ovarian Carcinogenesis: Revisited, Revised, and Expanded. Am. J. Pathol. 186, 733–747.

Kurman, R.J., Shih, I.-M., 2011. Molecular pathogenesis and extraovarian origin of epithelial ovarian cancer--shifting the paradigm. Hum. Pathol. 42, 918–931.

Kurman, R.J., Shih, I.-M., 2010. The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory. Am. J. Surg. Pathol. 34, 433–443.

La Vecchia, C., 2017. Ovarian cancer: epidemiology and risk factors. Eur. J. Cancer Prev. Off. J. Eur. Cancer Prev. Organ. ECP 26, 55–62.

Lambrechts, S., Smeets, D., Moisse, M., Braicu, E.I., Vanderstichele, A., Zhao, H., Van Nieuwenhuysen, E., Berns, E., Sehouli, J., Zeillinger, R., Darb-Esfahani, S., Cacsire Castillo-Tong, D., Lambrechts, D., Vergote, I., 2016. Genetic heterogeneity after first-line chemotherapy in high-grade serous ovarian cancer. Eur. J. Cancer Oxf. Engl. 1990 53, 51–64.

Langdon, S.P., Hirst, G.L., Miller, E.P., Hawkins, R.A., Tesdale, A.L., Smyth, J.F., Miller, W.R., 1994. The regulation of growth and protein expression by estrogen in vitro: a study of 8 human ovarian carcinoma cell lines. J. Steroid Biochem. Mol. Biol. 50, 131–135.

Laviolette, L.A., Garson, K., Macdonald, E.A., Senterman, M.K., Courville, K., Crane, C.A., Vanderhyden, B.C., 2010. 17beta-estradiol accelerates tumor onset and decreases survival in a transgenic mouse model of ovarian cancer. Endocrinology 151, 929–938.

Laviolette, L.A., Hodgkinson, K.M., Minhas, N., Perez-Iratxeta, C., Vanderhyden, B.C., 2014. 17β-estradiol upregulates GREB1 and accelerates ovarian tumor progression in vivo. Int. J. Cancer 135, 1072–1084.

Ledermann, J.A., Drew, Y., Kristeleit, R.S., 2016. Homologous recombination deficiency and ovarian cancer. Eur. J. Cancer Oxf. Engl. 1990 60, 49–58.

Lee, Y., Miron, A., Drapkin, R., Nucci, M.R., Medeiros, F., Saleemuddin, A., Garber, J., Birch, C., Mou, H., Gordon, R.W., Cramer, D.W., McKeon, F.D., Crum, C.P., 2007. A candidate precursor to serous carcinoma that originates in the distal fallopian tube. J. Pathol. 211, 26–35.

Lee, Y.J., Lee, S.W., Kim, K.R., Jung, K.H., Lee, J.W., Kim, Y.M., 2017. Pathologic findings at risk-reducing salpingo-oophorectomy (RRSO) in germline BRCA mutation carriers with breast cancer: significance of bilateral RRSO at the optimal age in germline BRCA mutation carriers. J. Gynecol. Oncol. 28, e3.

Leong, H.S., Galletta, L., Etemadmoghadam, D., George, J., Australian Ovarian Cancer Study, Köbel, M., Ramus, S.J., Bowtell, D., 2015. Efficient molecular subtype classification of high-grade serous ovarian cancer. J. Pathol. 236, 272–277.

Li, C.-F., Zhang, W.-G., Liu, M., Qiu, L.-W., Chen, X.-F., Lv, L., Mei, Z.-C., 2016. Aquaporin 9 inhibits hepatocellular carcinoma through up-regulating FOXO1 expression. Oncotarget 7, 44161–44170.

Li, D.-P., Du, C., Zhang, Z.-M., Li, G.-X., Yu, Z.-F., Wang, X., Li, P.-F., Cheng, C., Liu, Y.-P., Zhao, Y.-S., 2014. Breastfeeding and ovarian cancer risk: a systematic review and meta-analysis of 40 epidemiological studies. Asian Pac. J. Cancer Prev. APJCP 15, 4829–4837.

Li, L., Karanika, S., Yang, G., Wang, J., Park, S., Broom, B.M., Manyam, G.C., Wu, W., Luo, Y., Basourakos, S., Song, J.H., Gallick, G.E., Karantanos, T., Korentzelos, D., Azad, A.K., Kim, J., Corn, P.G., Aparicio, A.M., Logothetis, C.J., Troncoso, P., Heffernan, T., Toniatti, C., Lee, H.-S., Lee, J.-S., Zuo, X., Chang, W., Yin, J., Thompson, T.C., 2017. Androgen receptor inhibitor-induced "BRCAness" and PARP inhibition are synthetically lethal for castration-resistant prostate cancer. Sci. Signal. 10.

Li, M., Yu, X., 2015. The role of poly(ADP-ribosyl)ation in DNA damage response and cancer chemotherapy. Oncogene 34, 3349–3356.

Li, R., Wang, Y., Cheng, H., Liu, G., Cheng, T., Liu, Y., Liu, L., 2017. System modeling reveals the molecular mechanisms of HSC cell cycle alteration mediated by Maff and Egr3 under leukemia. BMC Syst. Biol. 11, 91.

Liang, B., Guo, Z., Li, Y., Liu, C., 2013. Elevated VEGF concentrations in ascites and serum predict adverse prognosis in ovarian cancer. Scand. J. Clin. Lab. Invest. 73, 309–314.

Liebner, D.A., Huang, K., Parvin, J.D., 2014. MMAD: microarray microdissection with analysis of differences is a computational tool for deconvoluting cell type-specific contributions from tissue samples. Bioinforma. Oxf. Engl. 30, 682–689.

Lord, C.J., Ashworth, A., 2017. PARP inhibitors: Synthetic lethality in the clinic. Science 355, 1152–1158.

Lord, C.J., Ashworth, A., 2016. BRCAness revisited. Nat. Rev. Cancer 16, 110–120.

Lord, C.J., Ashworth, A., 2013. Mechanisms of resistance to therapies targeting BRCA-mutant cancers. Nat. Med. 19, 1381–1388.

Lund, R.J., Huhtinen, K., Salmi, J., Rantala, J., Nguyen, E.V., Moulder, R., Goodlett, D.R., Lahesmaa, R., Carpén, O., 2017. DNA methylation and Transcriptome Changes Associated with Cisplatin Resistance in Ovarian Cancer. Sci. Rep. 7, 1469.

Luo, Y., Lee, M., Kim, H.S., Chung, H.H., Song, Y.S., 2016. Effect of neoadjuvant chemotherapy on platinum resistance in stage IIIC and IV epithelial ovarian cancer. Medicine (Baltimore) 95, e4797.

Lv, T., Song, K., Zhang, L., Li, W., Chen, Y., Diao, Y., Yao, Q., Liu, P., 2018. miRNA-34a decreases ovarian cancer cell proliferation and chemoresistance by targeting HDAC1. Biochem. Cell Biol. Biochim. Biol. Cell. 96, 663–671.

Macabelli, C.H., Ferreira, R.M., Gimenes, L.U., de Carvalho, N.A.T., Soares, J.G., Ayres, H., Ferraz, M.L., Watanabe, Y.F., Watanabe, O.Y., Sangalli, J.R., Smith, L.C., Baruselli, P.S., Meirelles, F.V., Chiaratti, M.R., 2014. Reference gene selection for gene expression analysis of oocytes collected from dairy cattle and buffaloes during winter and summer. PloS One 9, e93287.

Macintyre, G., Goranova, T.E., De Silva, D., Ennis, D., Piskorz, A.M., Eldridge, M., Sie, D., Lewsley, L.-A., Hanif, A., Wilson, C., Dowson, S., Glasspool, R.M., Lockley, M., Brockbank, E., Montes, A., Walther, A., Sundar, S., Edmondson, R., Hall, G.D., Clamp, A., Gourley, C., Hall, M., Fotopoulou, C., Gabra, H., Paul, J., Supernat, A., Millan, D., Hoyle, A., Bryson, G., Nourse, C., Mincarelli, L., Sanchez, L.N., Ylstra, B., Jimenez-Linan, M., Moore, L.,

Hofmann, O., Markowetz, F., McNeish, I.A., Brenton, J.D., 2018. Copy number signatures and mutational processes in ovarian carcinoma. Nat. Genet. 50, 1262–1270.

Mäenpää, J.U., n.d. Munasarjasyövän uusien lääkehoitojen mahdollisuudet. Duodecim Laaketieteellinen Aikakauskirja 2018.

Malpica, A., Deavers, M.T., Lu, K., Bodurka, D.C., Atkinson, E.N., Gershenson, D.M., Silva, E.G., 2004. Grading ovarian serous carcinoma using a two-tier system. Am. J. Surg. Pathol. 28, 496–504.

Manchanda, R., Menon, U., 2017. Setting the Threshold for Surgical Prevention in Women at Increased Risk of Ovarian Cancer: Int. J. Gynecol. Cancer 1.

Maniswami, R.R., Prashanth, S., Karanth, A.V., Koushik, S., Govindaraj, H., Mullangi, R., Rajagopal, S., Jegatheesan, S.K., 2018. PLK4: a link between centriole biogenesis and cancer. Expert Opin. Ther. Targets 22, 59–73.

MAQC Consortium, Shi, L., Reid, L.H., Jones, W.D., Shippy, R., Warrington, J.A., Baker, S.C., Collins, P.J., de Longueville, F., Kawasaki, E.S., Lee, K.Y., Luo, Y., Sun, Y.A., Willey, J.C., Setterquist, R.A., Fischer, G.M., Tong, W., Dragan, Y.P., Dix, D.J., Frueh, F.W., Goodsaid, F.M., Herman, D., Jensen, R.V., Johnson, C.D., Lobenhofer, E.K., Puri, R.K., Schrf, U., Thierry-Mieg, J., Wang, C., Wilson, M., Wolber, P.K., Zhang, Lu, Amur, S., Bao, W., Barbacioru, C.C., Lucas, A.B., Bertholet, V., Boysen, C., Bromley, B., Brown, D., Brunner, A., Canales, R., Cao, X.M., Cebula, T.A., Chen, J.J., Cheng, J., Chu, T.-M., Chudin, E., Corson, J., Corton, J.C., Croner, L.J., Davies, C., Davison, T.S., Delenstarr, G., Deng, X., Dorris, D., Eklund, A.C., Fan, X., Fang, H., Fulmer-Smentek, S., Fuscoe, J.C., Gallagher, K., Ge, W., Guo, L., Guo, X., Hager, J., Haje, P.K., Han, J., Han, T., Harbottle, H.C., Harris, S.C., Hatchwell, E., Hauser, C.A., Hester, S., Hong, H., Hurban, P., Jackson, S.A., Ji, H., Knight, C.R., Kuo, W.P., LeClerc, J.E., Levy, S., Li, Q.-Z., Liu, C., Liu, Y., Lombardi, M.J., Ma, Y., Magnuson, S.R., Maqsodi, B., McDaniel, T., Mei, N., Myklebost, O., Ning, B., Novoradovskaya, N., Orr, M.S., Osborn, T.W., Papallo, A., Patterson, T.A., Perkins, R.G., Peters, E.H., Peterson, R., Philips, K.L., Pine, P.S., Pusztai, L., Qian, F., Ren, H., Rosen, M., Rosenzweig, B.A., Samaha, R.R., Schena, M., Schroth, G.P., Shchegrova, S., Smith, D.D., Staedtler, F., Su, Z., Sun, H., Szallasi, Z., Tezak, Z., Thierry-Mieg, D., Thompson, K.L., Tikhonova, I., Turpaz, Y., Vallanat, B., Van, C., Walker, S.J., Wang, S.J., Wang, Y., Wolfinger, R., Wong, A., Wu, J., Xiao, C., Xie, Q., Xu, J., Yang, W., Zhang, Liang, Zhong, S., Zong, Y., Slikker, W., 2006. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. Nat. Biotechnol. 24, 1151–

Marchetti, C., De Felice, F., Palaia, I., Perniola, G., Musella, A., Musio, D., Muzii, L., Tombolini, V., Panici, P.B., 2014. Risk-reducing salpingo-oophorectomy: a meta-analysis on impact on ovarian cancer risk and all cause mortality in BRCA 1 and BRCA 2 mutation carriers. BMC Womens Health 14, 150.

Mardia, K.V., Li, Q., Hainsworth, T.J., 1992. On the Penrose hypothesis on fingerprint patterns. IMA J. Math. Appl. Med. Biol. 9, 289–294.

Mariani, M., McHugh, M., Petrillo, M., Sieber, S., He, S., Andreoli, M., Wu, Z., Fiedler, P., Scambia, G., Shahabi, S., Ferlini, C., 2014. HGF/c-Met axis drives cancer aggressiveness in the neo-adjuvant setting of ovarian cancer. Oncotarget 5, 4855–4867.

Markman, M., Bundy, B.N., Alberts, D.S., Fowler, J.M., Clark-Pearson, D.L., Carson, L.F., Wadler, S., Sickel, J., 2001. Phase III trial of standard-dose intravenous cisplatin plus paclitaxel versus moderately high-dose carboplatin followed by intravenous paclitaxel and intraperitoneal cisplatin in small-volume stage III ovarian carcinoma: an intergroup study of the Gynecologic Oncology Group, Southwestern Oncology Group, and Eastern Cooperative Oncology Group. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 19, 1001–1007.

Marth, C., Reimer, D., Zeimet, A.G., 2017. Front-line therapy of advanced epithelial ovarian cancer: standard treatment. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 28, viii36–viii39.

Martinez, A., Ngo, C., Leblanc, E., Gouy, S., Luyckx, M., Darai, E., Classe, J.M., Guyon, F., Pomel, C., Ferron, G., Filleron, T., Querleu, D., 2016. Surgical Complexity Impact on Survival After Complete Cytoreductive Surgery for Advanced Ovarian Cancer. Ann. Surg. Oncol. 23, 2515–2521.

Matsubayashi, H., Takaori, K., Morizane, C., Maguchi, H., Mizuma, M., Takahashi, H., Wada, K., Hosoi, H., Yachida, S., Suzuki, M., Usui, R., Furukawa, T., Furuse, J., Sato, T., Ueno, M., Kiyozumi, Y., Hijioka, S., Mizuno, N., Terashima, T., Mizumoto, M., Kodama, Y., Torishima, M., Kawaguchi, T., Ashida, R., Kitano, M., Hanada, K., Furukawa, M., Kawabe, K., Majima, Y., Shimosegawa, T., 2017. Familial pancreatic cancer: Concept, management and issues. World J. Gastroenterol. 23, 935–948.

Matsuo, K., Eno, M.L., Im, D.D., Rosenshein, N.B., 2010. Chemotherapy time interval and development of platinum and taxane resistance in ovarian, fallopian, and peritoneal carcinomas. Arch. Gynecol. Obstet. 281, 325–328.

Maxwell, G.L., Mutch, D.G., 2017. The promise of molecular staging for the future. Cancer 123, 728–730.

May, T., Comeau, R., Sun, P., Kotsopoulos, J., Narod, S.A., Rosen, B., Ghatage, P., 2017. A Comparison of Survival Outcomes in Advanced Serous Ovarian Cancer Patients Treated With Primary Debulking Surgery Versus Neoadjuvant Chemotherapy. Int. J. Gynecol. Cancer Off. J. Int. Gynecol. Cancer Soc. 27, 668–674.

McGuire, W.P., Hoskins, W.J., Brady, M.F., Kucera, P.R., Partridge, E.E., Look, K.Y., Clarke-Pearson, D.L., Davidson, M., 1996. Cyclophosphamide and cisplatin versus paclitaxel and cisplatin: a phase III randomized trial in patients with suboptimal stage III/IV ovarian cancer (from the Gynecologic Oncology Group). Semin. Oncol. 23, 40–47.

Menon, U., 2004. Ovarian cancer screening. CMAJ Can. Med. Assoc. J. J. Assoc. Medicale Can. 171, 323–324.

Menon, U., Griffin, M., Gentry-Maharaj, A., 2014. Ovarian cancer screening--current status, future directions. Gynecol. Oncol. 132, 490–495.

Mikami, M., 2014. Role of lymphadenectomy for ovarian cancer. J. Gynecol. Oncol. 25, 279–281.

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W., 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 266, 66–71.

Milliken, D., Scotton, C., Raju, S., Balkwill, F., Wilson, J., 2002. Analysis of chemokines and chemokine receptor expression in ovarian cancer ascites. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 8, 1108–1114.

Minig, L., Heitz, F., Cibula, D., Bakkum-Gamez, J.N., Germanova, A., Dowdy, S.C., Kalogera, E., Zapardiel, I., Lindemann, K., Harter, P., Scambia, G., Petrillo, M., Zorrero, C., Zanagnolo, V., Rebollo, J.M.C., du Bois, A., Fotopoulou, C., 2017. Patterns of Lymph Node Metastases in Apparent Stage I Low-Grade Epithelial Ovarian Cancer: A Multicenter Study. Ann. Surg. Oncol. 24, 2720–2726.

Mirza, M.R., Matulonis, U.A., 2017. Niraparib in Recurrent Ovarian Cancer. N. Engl. J. Med. 376, 801–802.

Mirza, M.R., Pignata, S., Ledermann, J.A., 2018. Latest clinical evidence and further development of PARP inhibitors in ovarian cancer. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 29, 1366–1376.

Mohammed, H., D'Santos, C., Serandour, A.A., Ali, H.R., Brown, G.D., Atkins, A., Rueda, O.M., Holmes, K.A., Theodorou, V., Robinson, J.L.L., Zwart, W., Saadi, A., Ross-Innes, C.S., Chin, S.-F., Menon, S., Stingl, J., Palmieri, C., Caldas, C., Carroll, J.S., 2013. Endogenous purification reveals GREB1 as a key estrogen receptor regulatory factor. Cell Rep. 3, 342–349.

Moore, K., Colombo, N., Scambia, G., Kim, B.-G., Oaknin, A., Friedlander, M., Lisyanskaya, A., Floquet, A., Leary, A., Sonke, G.S., Gourley, C., Banerjee, S., Oza, A., González-Martín, A., Aghajanian, C., Bradley, W., Mathews, C., Liu, J., Lowe, E.S., Bloomfield, R., DiSilvestro, P., 2018. Maintenance Olaparib in Patients with Newly Diagnosed Advanced Ovarian Cancer. N. Engl. J. Med. 379, 2495–2505.

Morris, L.G.T., Chan, T.A., 2015. Therapeutic targeting of tumor suppressor genes. Cancer 121, 1357–1368.

Morrison, J., Haldar, K., Kehoe, S., Lawrie, T.A., 2012. Chemotherapy versus surgery for initial treatment in advanced ovarian epithelial cancer. Cochrane Database Syst. Rev. CD005343.

Moynahan, M.E., Chiu, J.W., Koller, B.H., Jasin, M., 1999. Brca1 controls homology-directed DNA repair. Mol. Cell 4, 511–518.

Mutch, D.G., Prat, J., 2014. 2014 FIGO staging for ovarian, fallopian tube and peritoneal cancer. Gynecol. Oncol. 133, 401–404.

Nagy, J.A., Herzberg, K.T., Dvorak, J.M., Dvorak, H.F., 1993. Pathogenesis of malignant ascites formation: initiating events that lead to fluid accumulation. Cancer Res. 53, 2631–2643.

Nakonechny, Q.B., Gilks, C.B., 2016. Ovarian Cancer in Hereditary Cancer Susceptibility Syndromes. Surg. Pathol. Clin. 9, 189–199.

Ness, R.B., Cottreau, C., 1999. Possible role of ovarian epithelial inflammation in ovarian cancer. J. Natl. Cancer Inst. 91, 1459–1467.

Niemi, R.J., Saarelainen, S.K., Luukkaala, T.H., Mäenpää, J.U., 2017. Reliability of preoperative evaluation of postmenopausal ovarian tumors. J. Ovarian Res. 10, 15.

Kristoffersson, U., Kvist, A., Silfverberg, B., Borg, Å., Loman, N., 2018. BRCAsearch: written pre-test information and BRCA1/2 germline mutation testing in unselected patients with newly diagnosed breast cancer. Breast Cancer Res. Treat. 168, 117–126.

Nishida, N., Murakami, F., Higaki, K., 2016. Detection of serous precursor lesions in resected fallopian tubes from patients with benign diseases and a relatively low risk for ovarian cancer. Pathol. Int. 66, 337–342.

O'Connor, M.J., 2015. Targeting the DNA Damage Response in Cancer. Mol. Cell 60, 547–560.

Ohmoto, A., Yachida, S., 2017. Current status of poly(ADP-ribose) polymerase inhibitors and future directions. OncoTargets Ther. 10, 5195–5208.

O'Neil, N.J., Bailey, M.L., Hieter, P., 2017. Synthetic lethality and cancer. Nat. Rev. Genet. 18, 613–623.

Oronsky, B., Ray, C.M., Spira, A.I., Trepel, J.B., Carter, C.A., Cottrill, H.M., 2017. A brief review of the management of platinum-resistant-platinum-refractory ovarian cancer. Med. Oncol. Northwood Lond. Engl. 34, 103.

Osman, M.A., Elkady, M.S., Nasr, K.E., 2016. Weekly Paclitaxel Versus Three-Weekly Paclitaxel in Recurrent Platinum-Resistant Epithelial Ovarian and Peritoneal Cancers: A Phase III Study. Clin. Med. Insights Oncol. 10, 35–41.

Oza, A.M., Cook, A.D., Pfisterer, J., Embleton, A., Ledermann, J.A., Pujade-Lauraine, E., Kristensen, G., Carey, M.S., Beale, P., Cervantes, A., Park-Simon, T.-W., Rustin, G., Joly, F., Mirza, M.R., Plante, M., Quinn, M., Poveda, A., Jayson, G.C., Stark, D., Swart, A.M., Farrelly, L., Kaplan, R., Parmar, M.K.B., Perren, T.J., ICON7 trial investigators, 2015. Standard chemotherapy with or without bevacizumab for women with newly diagnosed ovarian cancer (ICON7): overall survival results of a phase 3 randomised trial. Lancet Oncol. 16, 928–936.

Ozben, T., 2006. Mechanisms and strategies to overcome multiple drug resistance in cancer. FEBS Lett. 580, 2903–2909.

Pascal, J.M., 2018. The comings and goings of PARP-1 in response to DNA damage. DNA Repair 71, 177–182.

Patch, A.-M., Christie, E.L., Etemadmoghadam, D., Garsed, D.W., George, J., Fereday, S., Nones, K., Cowin, P., Alsop, K., Bailey, P.J., Kassahn, K.S., Newell, F., Quinn, M.C.J., Kazakoff, S., Quek, K., Wilhelm-Benartzi, C., Curry, E., Leong, H.S., Australian Ovarian Cancer Study Group, Hamilton, A., Mileshkin, L., Au-Yeung, G., Kennedy, C., Hung, J., Chiew, Y.-E., Harnett, P., Friedlander, M., Quinn, M., Pyman, J., Cordner, S., O'Brien, P., Leditschke, J., Young, G., Strachan, K., Waring, P., Azar, W., Mitchell, C., Traficante, N., Hendley, J., Thorne, H., Shackleton, M., Miller, D.K., Arnau, G.M., Tothill, R.W., Holloway, T.P., Semple, T., Harliwong, I., Nourse, C., Nourbakhsh, E., Manning, S., Idrisoglu, S., Bruxner, T.J.C., Christ, A.N., Poudel, B., Holmes, O., Anderson, M., Leonard, C., Lonie, A., Hall, N., Wood, S., Taylor, D.F., Xu, Q., Fink, J.L., Waddell, Nick, Drapkin, R., Stronach, E., Gabra, H., Brown, R., Jewell, A., Nagaraj, S.H., Markham, E., Wilson, P.J., Ellul, J., McNally, O., Doyle, M.A., Vedururu, R., Stewart, C., Lengyel, E., Pearson, J.V., Waddell, Nicola, deFazio, A., Grimmond, S.M., Bowtell, D.D.L., 2015. Whole-genome characterization of chemoresistant ovarian cancer. Nature 521, 489–494.

Patwardhan, S., Gashler, A., Siegel, M.G., Chang, L.C., Joseph, L.J., Shows, T.B., Le Beau, M.M., Sukhatme, V.P., 1991. EGR3, a novel member of the Egr family of genes encoding immediate-early transcription factors. Oncogene 6, 917–928.

Pearce, C.L., Stram, D.O., Ness, R.B., Stram, D.A., Roman, L.D., Templeman, C., Lee, A.W., Menon, U., Fasching, P.A., McAlpine, J.N., Doherty, J.A., Modugno, F., Schildkraut, J.M., Rossing, M.A., Huntsman, D.G., Wu, A.H., Berchuck, A., Pike, M.C., Pharoah, P.D.P., 2015. Population distribution of lifetime risk of ovarian cancer in the United States. Cancer Epidemiol. Biomark. Prev. Publ. Am. Assoc. Cancer Res. Cosponsored Am. Soc. Prev. Oncol. 24, 671–676.

Perets, R., Wyant, G.A., Muto, K.W., Bijron, J.G., Poole, B.B., Chin, K.T., Chen, J.Y.H., Ohman, A.W., Stepule, C.D., Kwak, S., Karst, A.M., Hirsch, M.S., Setlur, S.R., Crum, C.P., Dinulescu, D.M., Drapkin, R., 2013. Transformation of the fallopian tube secretory epithelium leads to high-grade serous ovarian cancer in Brca;Tp53;Pten models. Cancer Cell 24, 751–765.

Perren, T.J., Swart, A.M., Pfisterer, J., Ledermann, J.A., Pujade-Lauraine, E., Kristensen, G., Carey, M.S., Beale, P., Cervantes, A., Kurzeder, C., du Bois, A., Sehouli, J., Kimmig, R., Stähle, A., Collinson, F., Essapen, S., Gourley, C., Lortholary, A., Selle, F., Mirza, M.R.,

Leminen, A., Plante, M., Stark, D., Qian, W., Parmar, M.K.B., Oza, A.M., ICON7 Investigators, 2011. A phase 3 trial of bevacizumab in ovarian cancer. N. Engl. J. Med. 365, 2484–2496.

Piek, J.M., van Diest, P.J., Zweemer, R.P., Jansen, J.W., Poort-Keesom, R.J., Menko, F.H., Gille, J.J., Jongsma, A.P., Pals, G., Kenemans, P., Verheijen, R.H., 2001. Dysplastic changes in prophylactically removed Fallopian tubes of women predisposed to developing ovarian cancer. J. Pathol. 195, 451–456.

Pignata, S., C Cecere, S., Du Bois, A., Harter, P., Heitz, F., 2017. Treatment of recurrent ovarian cancer. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 28, viii51–viii56.

Pommier, Y., Huang, S.N., Gao, R., Das, B.B., Murai, J., Marchand, C., 2014. Tyrosyl-DNA-phosphodiesterases (TDP1 and TDP2). DNA Repair 19, 114–129.

Povey, S., Lovering, R., Bruford, E., Wright, M., Lush, M., Wain, H., 2001. The HUGO Gene Nomenclature Committee (HGNC). Hum. Genet. 109, 678–680.

Praestegaard, C., Jensen, A., Jensen, S.M., Nielsen, T.S.S., Webb, P.M., Nagle, C.M., DeFazio, A., Australian Ovarian Cancer Study Group, Høgdall, E., Rossing, M.A., Doherty, J.A., Wicklund, K.G., Goodman, M.T., Modugno, F., Moysich, K., Ness, R.B., Edwards, R., Matsuo, K., Hosono, S., Goode, E.L., Winham, S.J., Fridley, B.L., Cramer, D.W., Terry, K.L., Schildkraut, J.M., Berchuck, A., Bandera, E.V., Paddock, L.E., Massuger, L.F., Wentzensen, N., Pharoah, P., Song, H., Whittemore, A., McGuire, V., Sieh, W., Rothstein, J., Anton-Culver, H., Ziogas, A., Menon, U., Gayther, S.A., Ramus, S.J., Gentry-Maharaj, A., Wu, A.H., Pearce, C.L., Pike, M., Lee, A.W., Sutphen, R., Chang-Claude, J., Risch, H.A., Kjaer, S.K., Ovarian Cancer Association Consortium, 2017. Cigarette smoking is associated with adverse survival among women with ovarian cancer: Results from a pooled analysis of 19 studies. Int. J. Cancer 140, 2422–2435.

Prakash, R., Zhang, Y., Feng, W., Jasin, M., 2015. Homologous recombination and human health: the roles of BRCA1, BRCA2, and associated proteins. Cold Spring Harb. Perspect. Biol. 7, a016600.

Prat, J., 2015. Ovarian, fallopian tube and peritoneal cancer staging: Rationale and explanation of new FIGO staging 2013. Best Pract. Res. Clin. Obstet. Gynaecol. 29, 858–869.

Prat, J., 2012. Ovarian carcinomas: five distinct diseases with different origins, genetic alterations, and clinicopathological features. Virchows Arch. Int. J. Pathol. 460, 237–249.

Press, J.Z., Wurz, K., Norquist, B.M., Lee, M.K., Pennil, C., Garcia, R., Welcsh, P., Goff, B.A., Swisher, E.M., 2010. Identification of a preneoplastic gene expression profile in tubal epithelium of BRCA1 mutation carriers. Neoplasia N. Y. N 12, 993–1002.

Pujade-Lauraine, E., Hilpert, F., Weber, B., Reuss, A., Poveda, A., Kristensen, G., Sorio, R., Vergote, I., Witteveen, P., Bamias, A., Pereira, D., Wimberger, P., Oaknin, A., Mirza, M.R., Follana, P., Bollag, D., Ray-Coquard, I., 2014. Bevacizumab combined with chemotherapy for platinum-resistant recurrent ovarian cancer: The AURELIA open-label randomized phase III trial. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 32, 1302–1308.

Pujade-Lauraine, E., Ledermann, J.A., Selle, F., Gebski, V., Penson, R.T., Oza, A.M., Korach, J., Huzarski, T., Poveda, A., Pignata, S., Friedlander, M., Colombo, N., Harter, P., Fujiwara, K., Ray-Coquard, I., Banerjee, S., Liu, J., Lowe, E.S., Bloomfield, R., Pautier, P., SOLO2/ENGOT-Ov21 investigators, 2017. Olaparib tablets as maintenance therapy in patients with platinum-sensitive, relapsed ovarian cancer and a BRCA1/2 mutation (SOLO2/ENGOT-Ov21): a double-blind, randomised, placebo-controlled, phase 3 trial. Lancet Oncol. 18, 1274–1284.

Pujade-Lauraine, E., Wagner, U., Aavall-Lundqvist, E., Gebski, V., Heywood, M., Vasey, P.A., Volgger, B., Vergote, I., Pignata, S., Ferrero, A., Sehouli, J., Lortholary, A., Kristensen, G., Jackisch, C., Joly, F., Brown, C., Le Fur, N., du Bois, A., 2010. Pegylated liposomal Doxorubicin and Carboplatin compared with Paclitaxel and Carboplatin for patients with platinum-sensitive ovarian cancer in late relapse. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 28, 3323–3329.

Qin, M., Jin, Y., Ma, L., Zhang, Y.-Y., Pan, L.-Y., 2018. The role of neoadjuvant chemotherapy followed by interval debulking surgery in advanced ovarian cancer: a systematic review and meta-analysis of randomized controlled trials and observational studies. Oncotarget 9, 8614–8628.

Qiu, W., Lu, H., Qi, Y., Wang, X., 2016. Dietary fat intake and ovarian cancer risk: a meta-analysis of epidemiological studies. Oncotarget 7, 37390–37406.

Ramalingam, P., 2016. Morphologic, Immunophenotypic, and Molecular Features of Epithelial Ovarian Cancer. Oncol. Williston Park N 30, 166–176.

Rasmussen, C.B., Kjaer, S.K., Albieri, V., Bandera, E.V., Doherty, J.A., Høgdall, E., Webb, P.M., Jordan, S.J., Rossing, M.A., Wicklund, K.G., Goodman, M.T., Modugno, F., Moysich, K.B., Ness, R.B., Edwards, R.P., Schildkraut, J.M., Berchuck, A., Olson, S.H., Kiemeney, L.A., Massuger, L.F.A.G., Narod, S.A., Phelan, C.M., Anton-Culver, H., Ziogas, A., Wu, A.H., Pearce, C.L., Risch, H.A., Jensen, A., , on behalf of the Ovarian Cancer Association Consortium, 2017. Pelvic Inflammatory Disease and the Risk of Ovarian Cancer and Borderline Ovarian Tumors: A Pooled Analysis of 13 Case-Control Studies. Am. J. Epidemiol. 185, 8–20.

Reuss, A., du Bois, A., Harter, P., Fotopoulou, C., Sehouli, J., Aletti, G., Guyon, F., Greggi, S., Mosgaard, B.J., Reinthaller, A., Hilpert, F., Schade-Brittinger, C., Chi, D.S., Mahner, S., 2019. TRUST: Trial of Radical Upfront Surgical Therapy in advanced ovarian cancer (ENGOT ov33/AGO-OVAR OP7). Int. J. Gynecol. Cancer Off. J. Int. Gynecol. Cancer Soc.

Ribatti, D., Ranieri, G., Annese, T., Nico, B., 2014. Aquaporins in cancer. Biochim. Biophys. Acta 1840, 1550–1553.

'Rice, M.S., Hankinson, S.E., Tworoger, S.S., 2014. Tubal ligation, hysterectomy, unilateral oophorectomy, and risk of ovarian cancer in the Nurses' Health Studies. Fertil. Steril. 102, 192-198.e3.

Risch, H.A., Howe, G.R., 1995. Pelvic inflammatory disease and the risk of epithelial ovarian cancer. Cancer Epidemiol. Biomark. Prev. Publ. Am. Assoc. Cancer Res. Cosponsored Am. Soc. Prev. Oncol. 4, 447–451.

Roarty, K., Pfefferle, A.D., Creighton, C.J., Perou, C.M., Rosen, J.M., 2017. Ror2-mediated alternative Wnt signaling regulates cell fate and adhesion during mammary tumor progression. Oncogene 36, 5958–5968.

Rojas, V., Hirshfield, K.M., Ganesan, S., Rodriguez-Rodriguez, L., 2016. Molecular Characterization of Epithelial Ovarian Cancer: Implications for Diagnosis and Treatment. Int. J. Mol. Sci. 17.

Ruderman, R., Pavone, M.E., 2017. Ovarian cancer in endometriosis: an update on the clinical and molecular aspects. Minerva Ginecol. 69, 286–294.

Sander Effron, S., Makvandi, M., Lin, L., Xu, K., Li, S., Lee, H., Hou, C., Pryma, D.A., Koch,

C., Mach, R.H., 2017. PARP-1 Expression Quantified by [18F]FluorThanatrace: A Biomarker of Response to PARP Inhibition Adjuvant to Radiation Therapy. Cancer Biother. Radiopharm. 32, 9–15.

Sankala, H., Vaughan, C., Wang, J., Deb, S., Graves, P.R., 2011. Upregulation of the mitochondrial transport protein, Tim50, by mutant p53 contributes to cell growth and chemoresistance. Arch. Biochem. Biophys. 512, 52–60.

Sato, S., Itamochi, H., 2014. Neoadjuvant chemotherapy in advanced ovarian cancer: latest results and place in therapy. Ther. Adv. Med. Oncol. 6, 293–304.

Scaletta, G., Plotti, F., Luvero, D., Capriglione, S., Montera, R., Miranda, A., Lopez, S., Terranova, C., De Cicco Nardone, C., Angioli, R., 2017. The role of novel biomarker HE4 in the diagnosis, prognosis and follow-up of ovarian cancer: a systematic review. Expert Rev. Anticancer Ther. 17, 827–839.

Schiewer, M.J., Goodwin, J.F., Han, S., Brenner, J.C., Augello, M.A., Dean, J.L., Liu, F., Planck, J.L., Ravindranathan, P., Chinnaiyan, A.M., McCue, P., Gomella, L.G., Raj, G.V., Dicker, A.P., Brody, J.R., Pascal, J.M., Centenera, M.M., Butler, L.M., Tilley, W.D., Feng, F.Y., Knudsen, K.E., 2012. Dual roles of PARP-1 promote cancer growth and progression. Cancer Discov. 2, 1134–1149.

Schiewer, M.J., Mandigo, A.C., Gordon, N., Huang, F., Gaur, S., de Leeuw, R., Zhao, S.G., Evans, J., Han, S., Parsons, T., Birbe, R., McCue, P., McNair, C., Chand, S.N., Cendon-Florez, Y., Gallagher, P., McCann, J.J., Poudel Neupane, N., Shafi, A.A., Dylgjeri, E., Brand, L.J., Visakorpi, T., Raj, G.V., Lallas, C.D., Trabulsi, E.J., Gomella, L.G., Dicker, A.P., Kelly, W.K., Leiby, B.E., Knudsen, B., Feng, F.Y., Knudsen, K.E., 2018. PARP-1 regulates DNA repair factor availability. EMBO Mol. Med. 10.

Schlacher, K., Christ, N., Siaud, N., Egashira, A., Wu, H., Jasin, M., 2011. Double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11. Cell 145, 529–542.

Sehouli, J. (Ed.), 2014. Current treatment of ovarian cancer, 1st ed. ed, UNI-MED Science. UNI-MED-Verl, Bremen.

Seidman, J.D., Horkayne-Szakaly, I., Haiba, M., Boice, C.R., Kurman, R.J., Ronnett, B.M., 2004a. The histologic type and stage distribution of ovarian carcinomas of surface epithelial

origin. Int. J. Gynecol. Pathol. Off. J. Int. Soc. Gynecol. Pathol. 23, 41–44.

Seidman, J.D., Kurman, R.J., 2000. Ovarian serous borderline tumors: a critical review of the literature with emphasis on prognostic indicators. Hum. Pathol. 31, 539–557.

Seidman, J.D., Soslow, R.A., Vang, R., Berman, J.J., Stoler, M.H., Sherman, M.E., Oliva, E., Kajdacsy-Balla, A., Berman, D.M., Copeland, L.J., 2004b. Borderline ovarian tumors: diverse contemporary viewpoints on terminology and diagnostic criteria with illustrative images. Hum. Pathol. 35, 918–933.

Severson, T.M., Peeters, J., Majewski, I., Michaut, M., Bosma, A., Schouten, P.C., Chin, S.-F., Pereira, B., Goldgraben, M.A., Bismeijer, T., Kluin, R.J.C., Muris, J.J.F., Jirström, K., Kerkhoven, R.M., Wessels, L., Caldas, C., Bernards, R., Simon, I.M., Linn, S., 2015. BRCA1-like signature in triple negative breast cancer: Molecular and clinical characterization reveals subgroups with therapeutic potential. Mol. Oncol. 9, 1528–1538.

Shaw, R.J., 2009. Tumor suppression by LKB1: SIK-ness prevents metastasis. Sci. Signal. 2, pe55.

Shen, F., Zhang, X., Zhang, Y., Ding, J., Chen, Q., 2017. Hormone receptors expression in ovarian cancer taking into account menopausal status: a retrospective study in Chinese population. Oncotarget 8, 84019–84027.

Shen, J., Zhao, W., Ju, Z., Wang, L., Peng, Y., Labrie, M., Yap, T.A., Mills, G.B., Peng, G., 2019. PARPi Triggers the STING-Dependent Immune Response and Enhances the Therapeutic Efficacy of Immune Checkpoint Blockade Independent of BRCAness. Cancer Res. 79, 311–319.

Shih, I.-M., Kurman, R.J., 2004. Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. Am. J. Pathol. 164, 1511–1518.

Shimizu, Y., 2004. [Validity of complete paraaortic and pelvic lymphadenectomy in apparent stage I (T1) epithelial ovarian carcinoma]. Nihon Rinsho Jpn. J. Clin. Med. 62 Suppl 10, 570–577.

Siddiqui, G.K., Maclean, A.B., Elmasry, K., Wong te Fong, A., Morris, R.W., Rashid, M., Begent, R.H.J., Boxer, G.M., 2011. Immunohistochemical expression of VEGF predicts response to platinum based chemotherapy in patients with epithelial ovarian cancer.

Angiogenesis 14, 155–161.

Siegel, R.L., Miller, K.D., Jemal, A., 2015. Cancer statistics, 2015. CA. Cancer J. Clin. 65, 5–29.

Silverberg, S.G., Bell, D.A., Kurman, R.J., Seidman, J.D., Prat, J., Ronnett, B.M., Copeland, L., Silva, E., Gorstein, F., Young, R.H., 2004. Borderline ovarian tumors: key points and workshop summary. Hum. Pathol. 35, 910–917.

Simin, J., Tamimi, R., Lagergren, J., Adami, H.-O., Brusselaers, N., 2017. Menopausal hormone therapy and cancer risk: An overestimated risk? Eur. J. Cancer Oxf. Engl. 1990 84, 60–68.

Singh, N., McCluggage, W.G., Gilks, C.B., 2017. High-grade serous carcinoma of tubo-ovarian origin: recent developments. Histopathology 71, 339–356.

Skates, S.J., Greene, M.H., Buys, S.S., Mai, P.L., Brown, P., Piedmonte, M., Rodriguez, G., Schorge, J.O., Sherman, M., Daly, M.B., Rutherford, T., Brewster, W.R., O'Malley, D.M., Partridge, E., Boggess, J., Drescher, C.W., Isaacs, C., Berchuck, A., Domchek, S., Davidson, S.A., Edwards, R., Elg, S.A., Wakeley, K., Phillips, K.-A., Armstrong, D., Horowitz, I., Fabian, C.J., Walker, J., Sluss, P.M., Welch, W., Minasian, L., Horick, N.K., Kasten, C.H., Nayfield, S., Alberts, D., Finkelstein, D.M., Lu, K.H., 2017. Early Detection of Ovarian Cancer using the Risk of Ovarian Cancer Algorithm with Frequent CA125 Testing in Women at Increased Familial Risk - Combined Results from Two Screening Trials. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 23, 3628–3637.

Skates, S.J., Xu, F.J., Yu, Y.H., Sjövall, K., Einhorn, N., Chang, Y., Bast, R.C., Knapp, R.C., 1995. Toward an optimal algorithm for ovarian cancer screening with longitudinal tumor markers. Cancer 76, 2004–2010.

Skirnisdottir, I., Seidal, T., Åkerud, H., 2016. The relationship of the angiogenesis regulators VEGF-A, VEGF-R1 and VEGF-R2 to p53 status and prognostic factors in epithelial ovarian carcinoma in FIGO-stages I-II. Int. J. Oncol. 48, 998–1006.

Smyth, G.K., 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3, Article3.

Soini, T., Hurskainen, R., Grénman, S., Mäenpää, J., Paavonen, J., Pukkala, E., 2016. Impact

of levonorgestrel-releasing intrauterine system use on the cancer risk of the ovary and fallopian tube. Acta Oncol. Stockh. Swed. 55, 1281–1284.

Stewart, L.M., Spilsbury, K., Jordan, S., Stewart, C., Holman, C.D.J., Powell, A., Reekie, J., Cohen, P., 2018. Risk of high-grade serous ovarian cancer associated with pelvic inflammatory disease, parity and breast cancer. Cancer Epidemiol. 55, 110–116.

Suh, D.H., Kim, M., Lee, K.H., Eom, K.Y., Kjeldsen, M.K., Mirza, M.R., Kim, J.W., 2018. Major clinical research advances in gynecologic cancer in 2017. J. Gynecol. Oncol. 29, e31.

Sumithra, B., Saxena, U., Das, A.B., 2016. Alternative splicing within the Wnt signaling pathway: role in cancer development. Cell. Oncol. Dordr. 39, 1–13.

Tan, D.S.P., Rothermundt, C., Thomas, K., Bancroft, E., Eeles, R., Shanley, S., Ardern-Jones, A., Norman, A., Kaye, S.B., Gore, M.E., 2008. "BRCAness" syndrome in ovarian cancer: a case-control study describing the clinical features and outcome of patients with epithelial ovarian cancer associated with BRCA1 and BRCA2 mutations. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 26, 5530–5536.

Tangjitgamol, S., Manusirivithaya, S., Laopaiboon, M., Lumbiganon, P., Bryant, A., 2016. Interval debulking surgery for advanced epithelial ovarian cancer. Cochrane Database Syst. Rev. CD006014.

Tate, S., Kato, K., Nishikimi, K., Matsuoka, A., Shozu, M., 2017. Survival and safety associated with aggressive surgery for stage III/IV epithelial ovarian cancer: A single institution observation study. Gynecol. Oncol. 147, 73–80.

Taylor, K.N., Eskander, R.N., 2018. PARP Inhibitors in Epithelial Ovarian Cancer. Recent Patents Anticancer Drug Discov. 13, 145–158.

Temkin, S.M., Bergstrom, J., Samimi, G., Minasian, L., 2017. Ovarian Cancer Prevention in High-risk Women. Clin. Obstet. Gynecol. 60, 738–757.

Tetreault, M.-P., Yang, Y., Katz, J.P., 2013. Krüppel-like factors in cancer. Nat. Rev. Cancer 13, 701–713.

Thibault, B., Castells, M., Delord, J.-P., Couderc, B., 2014. Ovarian cancer microenvironment: implications for cancer dissemination and chemoresistance acquisition.

Cancer Metastasis Rev. 33, 17–39.

Tingulstad, S., Hagen, B., Skjeldestad, F.E., Onsrud, M., Kiserud, T., Halvorsen, T., Nustad, K., 1996. Evaluation of a risk of malignancy index based on serum CA125, ultrasound findings and menopausal status in the pre-operative diagnosis of pelvic masses. Br. J. Obstet. Gynaecol. 103, 826–831.

Tone, A.A., Begley, H., Sharma, M., Murphy, J., Rosen, B., Brown, T.J., Shaw, P.A., 2008. Gene expression profiles of luteal phase fallopian tube epithelium from BRCA mutation carriers resemble high-grade serous carcinoma. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 14, 4067–4078.

Toss, A., Tomasello, C., Razzaboni, E., Contu, G., Grandi, G., Cagnacci, A., Schilder, R.J., Cortesi, L., 2015. Hereditary ovarian cancer: not only BRCA 1 and 2 genes. BioMed Res. Int. 2015, 341723.

Trimbos, J.B., Parmar, M., Vergote, I., Guthrie, D., Bolis, G., Colombo, N., Vermorken, J.B., Torri, V., Mangioni, C., Pecorelli, S., Lissoni, A., Swart, A.M., International Collaborative Ovarian Neoplasm 1, European Organisation for Research and Treatment of Cancer Collaborators-Adjuvant ChemoTherapy un Ovarian Neoplasm, 2003. International Collaborative Ovarian Neoplasm trial 1 and Adjuvant ChemoTherapy In Ovarian Neoplasm trial: two parallel randomized phase III trials of adjuvant chemotherapy in patients with early-stage ovarian carcinoma. J. Natl. Cancer Inst. 95, 105–112.

Tschernichovsky, R., Goodman, A., 2017. Risk-Reducing Strategies for Ovarian Cancer in BRCA Mutation Carriers: A Balancing Act. The Oncologist 22, 450–459.

Turner, N., Tutt, A., Ashworth, A., 2004. Hallmarks of "BRCAness" in sporadic cancers. Nat. Rev. Cancer 4, 814–819.

Valladares, M., Plaza-Parrochia, F., Lépez, M., López, D., Gabler, F., Gayan, P., Selman, A., Vega, M., Romero, C., 2017. Effect of estradiol on the expression of angiogenic factors in epithelial ovarian cancer. Histol. Histopathol. 32, 1187–1196.

van Amerongen, R., 2012. Alternative Wnt pathways and receptors. Cold Spring Harb. Perspect. Biol. 4.

van der Steen, S.C.H.A., Bulten, J., Van de Vijver, K.K., van Kuppevelt, T.H., Massuger,

L.F.A.G., 2017. Changes in the Extracellular Matrix Are Associated With the Development of Serous Tubal Intraepithelial Carcinoma Into High-Grade Serous Carcinoma. Int. J. Gynecol. Cancer Off. J. Int. Gynecol. Cancer Soc. 27, 1072–1081.

van Driel, W.J., Koole, S.N., Sonke, G.S., 2018. Hyperthermic Intraperitoneal Chemotherapy in Ovarian Cancer. N. Engl. J. Med. 378, 1363–1364.

Vasey, P.A., Atkinson, R., Coleman, R., Crawford, M., Cruickshank, M., Eggleton, P., Fleming, D., Graham, J., Parkin, D., Paul, J., Reed, N.S., Kaye, S.B., 2001. Docetaxel-carboplatin as first line chemotherapy for epithelial ovarian cancer. Br. J. Cancer 84, 170–178.

Venkitaraman, A.R., 2002. Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell 108, 171–182.

Vergote, I., Tropé, C.G., Amant, F., Kristensen, G.B., Ehlen, T., Johnson, N., Verheijen, R.H.M., van der Burg, M.E.L., Lacave, A.J., Panici, P.B., Kenter, G.G., Casado, A., Mendiola, C., Coens, C., Verleye, L., Stuart, G.C.E., Pecorelli, S., Reed, N.S., European Organization for Research and Treatment of Cancer-Gynaecological Cancer Group, NCIC Clinical Trials Group, 2010. Neoadjuvant chemotherapy or primary surgery in stage IIIC or IV ovarian cancer. N. Engl. J. Med. 363, 943–953.

Verkman, A.S., Ruiz-Ederra, J., Levin, M.H., 2008. Functions of aquaporins in the eye. Prog. Retin. Eye Res. 27, 420–433.

Villegas-Ruiz, V., Moreno, J., Jacome-Lopez, K., Zentella-Dehesa, A., Juarez-Mendez, S., 2016. Quality Control Usage in High-Density Microarrays Reveals Differential Gene Expression Profiles in Ovarian Cancer. Asian Pac. J. Cancer Prev. APJCP 17, 2519–2525.

Wain, H.M., Bruford, E.A., Lovering, R.C., Lush, M.J., Wright, M.W., Povey, S., 2002. Guidelines for human gene nomenclature. Genomics 79, 464–470.

Walker, J.L., Powell, C.B., Chen, L.-M., Carter, J., Bae Jump, V.L., Parker, L.P., Borowsky, M.E., Gibb, R.K., 2015. Society of Gynecologic Oncology recommendations for the prevention of ovarian cancer. Cancer 121, 2108–2120.

Walsh, T., Casadei, S., Lee, M.K., Pennil, C.C., Nord, A.S., Thornton, A.M., Roeb, W., Agnew, K.J., Stray, S.M., Wickramanayake, A., Norquist, B., Pennington, K.P., Garcia, R.L.,

King, M.-C., Swisher, E.M., 2011. Mutations in 12 genes for inherited ovarian, fallopian tube, and peritoneal carcinoma identified by massively parallel sequencing. Proc. Natl. Acad. Sci. U. S. A. 108, 18032–18037.

Wang, B., Shen, A., Ouyang, X., Zhao, G., Du, Z., Huo, W., Zhang, T., Wang, Y., Yang, C., Dong, P., Watari, H., Pfeffer, L.M., Yue, J., 2017. KLF4 expression enhances the efficacy of chemotherapy drugs in ovarian cancer cells. Biochem. Biophys. Res. Commun. 484, 486–492.

Wang, F., Chang, J.T.-H., Zhang, Z., Morrison, G., Nath, A., Bhutra, S., Huang, R.S., 2017. Discovering drugs to overcome chemoresistance in ovarian cancers based on the cancer genome atlas tumor transcriptome profile. Oncotarget 8, 115102–115113.

Wang, H., Xu, T., Zheng, L., Li, G., 2018. Angiogenesis Inhibitors for the Treatment of Ovarian Cancer: An Updated Systematic Review and Meta-analysis of Randomized Controlled Trials. Int. J. Gynecol. Cancer Off. J. Int. Gynecol. Cancer Soc. 28, 903–914.

Wang, J., Gao, H., Liu, G., Gu, L., Yang, C., Zhang, F., Liu, T., 2018. Tumor necrosis factor α-induced protein 8 expression as a predictor of prognosis and resistance in patients with advanced ovarian cancer treated with neoadjuvant chemotherapy. Hum. Pathol. 82, 239–248.

Wang, Y., Ngo, V.N., Marani, M., Yang, Y., Wright, G., Staudt, L.M., Downward, J., 2010. Critical role for transcriptional repressor Snail2 in transformation by oncogenic RAS in colorectal carcinoma cells. Oncogene 29, 4658–4670.

Watkins, J.A., Irshad, S., Grigoriadis, A., Tutt, A.N.J., 2014. Genomic scars as biomarkers of homologous recombination deficiency and drug response in breast and ovarian cancers. Breast Cancer Res. BCR 16, 211.

Webb, P.M., Jordan, S.J., 2017. Epidemiology of epithelial ovarian cancer. Best Pract. Res. Clin. Obstet. Gynaecol. 41, 3–14.

Wei, S.U., Li, H., Zhang, B., 2016. The diagnostic value of serum HE4 and CA-125 and ROMA index in ovarian cancer. Biomed. Rep. 5, 41–44.

Wei, W., Chen, Y., Xu, J., Zhou, Y., Bai, X., Yang, M., Zhu, J., 2018. Identification of Biomarker for Cutaneous Squamous Cell Carcinoma Using Microarray Data Analysis. J.

Cancer 9, 400–406.

Wei, W., Li, Y., Lv, S., Zhang, C., Tian, Y., 2016. PARP-1 may be involved in angiogenesis in epithelial ovarian cancer. Oncol. Lett. 12, 4561–4567.

Weidle, U.H., Birzele, F., Kollmorgen, G., Rueger, R., 2016. Mechanisms and Targets Involved in Dissemination of Ovarian Cancer. Cancer Genomics Proteomics 13, 407–423.

Wein, M.N., Foretz, M., Fisher, D.E., Xavier, R.J., Kronenberg, H.M., 2018. Salt-Inducible Kinases: Physiology, Regulation by cAMP, and Therapeutic Potential. Trends Endocrinol. Metab. TEM 29, 723–735.

Wiegand, K.C., Shah, S.P., Al-Agha, O.M., Zhao, Y., Tse, K., Zeng, T., Senz, J., McConechy, M.K., Anglesio, M.S., Kalloger, S.E., Yang, W., Heravi-Moussavi, A., Giuliany, R., Chow, C., Fee, J., Zayed, A., Prentice, L., Melnyk, N., Turashvili, G., Delaney, A.D., Madore, J., Yip, S., McPherson, A.W., Ha, G., Bell, L., Fereday, S., Tam, A., Galletta, L., Tonin, P.N., Provencher, D., Miller, D., Jones, S.J.M., Moore, R.A., Morin, G.B., Oloumi, A., Boyd, N., Aparicio, S.A., Shih, I.-M., Mes-Masson, A.-M., Bowtell, D.D., Hirst, M., Gilks, B., Marra, M.A., Huntsman, D.G., 2010. ARID1A mutations in endometriosis-associated ovarian carcinomas. N. Engl. J. Med. 363, 1532–1543.

Wilson, M.K., Pujade-Lauraine, E., Aoki, D., Mirza, M.R., Lorusso, D., Oza, A.M., du Bois, A., Vergote, I., Reuss, A., Bacon, M., Friedlander, M., Gallardo-Rincon, D., Joly, F., Chang, S.-J., Ferrero, A.M., Edmondson, R.J., Wimberger, P., Maenpaa, J., Gaffney, D., Zang, R., Okamoto, A., Stuart, G., Ochiai, K., participants of the Fifth Ovarian Cancer Consensus Conference, 2017. Fifth Ovarian Cancer Consensus Conference of the Gynecologic Cancer InterGroup: recurrent disease. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 28, 727–732.

Wimberger, P., Lehmann, N., Kimmig, R., Burges, A., Meier, W., Du Bois, A., Arbeitsgemeinschaft Gynaekologische Onkologie Ovarian Cancer Study Group, 2007. Prognostic factors for complete debulking in advanced ovarian cancer and its impact on survival. An exploratory analysis of a prospectively randomized phase III study of the Arbeitsgemeinschaft Gynaekologische Onkologie Ovarian Cancer Study Group (AGO-OVAR). Gynecol. Oncol. 106, 69–74.

Wright, A.A., Bohlke, K., Armstrong, D.K., Bookman, M.A., Cliby, W.A., Coleman, R.L., Dizon, D.S., Kash, J.J., Meyer, L.A., Moore, K.N., Olawaiye, A.B., Oldham, J., Salani, R., Sparacio, D., Tew, W.P., Vergote, I., Edelson, M.I., 2016. Neoadjuvant Chemotherapy for

Newly Diagnosed, Advanced Ovarian Cancer: Society of Gynecologic Oncology and American Society of Clinical Oncology Clinical Practice Guideline. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 34, 3460–3473.

Wu, A.H., Pearce, C.L., Lee, A.W., Tseng, C., Jotwani, A., Patel, P., Pike, M.C., 2017. Timing of births and oral contraceptive use influences ovarian cancer risk: Timing of births influences ovarian cancer risk. Int. J. Cancer 141, 2392–2399.

Wu, D., Yang, H., Winham, S.J., Natanzon, Y., Koestler, D.C., Luo, T., Fridley, B.L., Goode, E.L., Zhang, Y., Cui, Y., 2018. Mediation analysis of alcohol consumption, DNA methylation, and epithelial ovarian cancer. J. Hum. Genet. 63, 339–348.

Yamauchi, H., Takei, J., 2018. Management of hereditary breast and ovarian cancer. Int. J. Clin. Oncol. 23, 45–51.

Yanaranop, M., Anakrat, V., Siricharoenthai, S., Nakrangsee, S., Thinkhamrop, B., 2017. Is the Risk of Ovarian Malignancy Algorithm Better Than Other Tests for Predicting Ovarian Malignancy in Women with Pelvic Masses? Gynecol. Obstet. Invest. 82, 47–53.

Yang, J.H., Yan, C.X., Chen, X.J., Zhu, Y.S., 2011. Expression of aquaglyceroporins in epithelial ovarian tumours and their clinical significance. J. Int. Med. Res. 39, 702–711.

Yang, L., Xie, N., Huang, J., Huang, H., Xu, S., Wang, Z., Cai, J., 2018. SIK1-LNC represses the proliferative, migrative, and invasive abilities of lung cancer cells. OncoTargets Ther. 11, 4197–4206.

Yan-Hong, H., Jing, L., Hong, L., Shan-Shan, H., Yan, L., Ju, L., 2015. Association between alcohol consumption and the risk of ovarian cancer: a meta-analysis of prospective observational studies. BMC Public Health 15, 223.

Young, R.C., Decker, D.G., Wharton, J.T., Piver, M.S., Sindelar, W.F., Edwards, B.K., Smith, J.P., 1983. Staging laparotomy in early ovarian cancer. JAMA 250, 3072–3076.

Yu, X., Chen, Y., Tian, R., Li, J., Li, H., Lv, T., Yao, Q., 2017. miRNA-21 enhances chemoresistance to cisplatin in epithelial ovarian cancer by negatively regulating PTEN. Oncol. Lett. 14, 1807–1810.

Zakhour, M., Danovitch, Y., Lester, J., Rimel, B.J., Walsh, C.S., Li, A.J., Karlan, B.Y., Cass,

I., 2016. Occult and subsequent cancer incidence following risk-reducing surgery in BRCA mutation carriers. Gynecol. Oncol. 143, 231–235.

Zeng, R., Huang, J., Zhong, M.-Z., Li, L., Yang, G., Liu, L., Wu, Y., Yao, X., Shi, J., Wu, Z., 2016. Multiple Roles of WNT5A in Breast Cancer. Med. Sci. Monit. Int. Med. J. Exp. Clin. Res. 22, 5058–5067.

Zhang, H., Liu, T., Zhang, Z., Payne, S.H., Zhang, Bai, McDermott, J.E., Zhou, J.-Y., Petyuk, V.A., Chen, Li, Ray, D., Sun, S., Yang, F., Chen, Lijun, Wang, J., Shah, P., Cha, S.W., Aiyetan, P., Woo, S., Tian, Y., Gritsenko, M.A., Clauss, T.R., Choi, C., Monroe, M.E., Thomas, S., Nie, S., Wu, C., Moore, R.J., Yu, K.-H., Tabb, D.L., Fenyö, D., Bafna, V., Wang, Y., Rodriguez, H., Boja, E.S., Hiltke, T., Rivers, R.C., Sokoll, L., Zhu, H., Shih, I.-M., Cope, L., Pandey, A., Zhang, Bing, Snyder, M.P., Levine, D.A., Smith, R.D., Chan, D.W., Rodland, K.D., CPTAC Investigators, 2016. Integrated Proteogenomic Characterization of Human High-Grade Serous Ovarian Cancer. Cell 166, 755–765.

Zhang, L., Nadeem, L., Connor, K., Xu, G., 2016. Mechanisms and Therapeutic Targets of microRNA-associated Chemoresistance in Epithelial Ovarian Cancer. Curr. Cancer Drug Targets 16, 429–441.

Zhang, M., Liu, G., Xue, F., Edwards, R., Sood, A.K., Zhang, W., Yang, D., 2016. Copy number deletion of RAD50 as predictive marker of BRCAness and PARP inhibitor response in BRCA wild type ovarian cancer. Gynecol. Oncol. 141, 57–64.

Zhang, Q., Burdette, J.E., Wang, J.-P., 2014. Integrative network analysis of TCGA data for ovarian cancer. BMC Syst. Biol. 8, 1338.

Zhang, W., Geiman, D.E., Shields, J.M., Dang, D.T., Mahatan, C.S., Kaestner, K.H., Biggs, J.R., Kraft, A.S., Yang, V.W., 2000. The gut-enriched Kruppel-like factor (Kruppel-like factor 4) mediates the transactivating effect of p53 on the p21WAF1/Cip1 promoter. J. Biol. Chem. 275, 18391–18398.

Zhang, X., Chen, J., Sun, L., Xu, Y., 2018. SIRT1 deacetylates KLF4 to activate Claudin-5 transcription in ovarian cancer cells. J. Cell. Biochem. 119, 2418–2426.

Zhao, H., Sun, Q., Li, L., Zhou, J., Zhang, C., Hu, T., Zhou, X., Zhang, L., Wang, B., Li, B., Zhu, T., Li, H., 2019. High Expression Levels of AGGF1 and MFAP4 Predict Primary Platinum-Based Chemoresistance and are Associated with Adverse Prognosis in Patients

with Serous Ovarian Cancer. J. Cancer 10, 397–407.

Zhao, T., Hu, W., 2016. CA125 and HE4: Measurement Tools for Ovarian Cancer. Gynecol. Obstet. Invest. 81, 430–435.

Zheng, X., Chen, S., Li, L., Liu, Xiaohua, Liu, Xiaomin, Dai, S., Zhang, P., Lu, H., Lin, Z., Yu, Y., Li, G., 2018. Evaluation of HE4 and TTR for diagnosis of ovarian cancer: Comparison with CA-125. J. Gynecol. Obstet. Hum. Reprod. 47, 227–230.

Zhou, Z., Zeng, F., Yuan, J., Tang, J., Colditz, G.A., Tworoger, S.S., Trabert, B., Su, X., 2017. Pelvic inflammatory disease and the risk of ovarian cancer: a meta-analysis. Cancer Causes Control CCC 28, 415–428.

Zhu, X., Gu, J., Qian, H., 2018. Esculetin Attenuates the Growth of Lung Cancer by Downregulating Wnt Targeted Genes and Suppressing NF-κB. Arch. Bronconeumol. 54, 128–133.

10 ORIGINAL PUBLICATIONS

Publication I Veskimäe, K., Staff, S., Tabaro, F , Nykter, M. , Isola, J., Mäenpää, J. Microarray analysis of differentially expressed genes in ovarian and fallopian tube epithelium from risk-reducing salpingo-oophorectomies. Genes Chromosomes Cancer. 2015 May;54(5):276-87

Publication II Veskimäe, K., Staff, S., Grönholm, A, Pesu, M., Laaksonen, M., Nykter, M., Isola, J. and Mäenpää, J. Assessment of PARP protein expression in epithelial ovarian cancer by ELISA pharmacodynamic assay and immunohistochemistry. Tumour Biol. 2016 Sep;37(9):11991-11999

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PUBLICATION

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Microarray analysis of differentially expressed genes in ovarian and fallopian tube epithelium from risk-reducing salpingo-oophorectomies

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Microarray Analysis of Differentially Expressed Genes in Ovarian and Fallopian Tube Epithelium From Risk-Reducing Salpingo-Oophorectomies

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Mutations in the *BRCA1* and *BRCA2* genes confer an increased lifetime risk for breast and ovarian cancer. Ovarian cancer risk can be decreased by risk-reducing salpingo-oophorectomy (RRSO). Studies on RRSO material have altered the paradigm of serous ovarian cancer pathogenesis. The purpose of this study was to identify candidate genes possibly involved in the pathogenesis of serous ovarian cancer by carrying out a microarray analysis of differentially expressed genes in *BRCA1/2*- mutation positive ovarian and fallopian tube epithelium derived from RRSO surgery. Freshly frozen ovarian and fallopian tube samples from nine *BRCA1/2* mutation carriers scheduled for RRSO were prospectively collected together with five mutation-negative control patients undergoing salpingo-oophorectomy for benign indications. Microarray analysis of genome-wide gene expression was performed on ovarian and fallopian tube samples from the *BRCA1/2* and control patients. The validation of microarray data was performed by quantitative real-time polymerase chain reaction (qRT-PCR) in selected cases of RRSO samples and also in high grade serous carcinoma samples collected from patients with a *BRCA* phenotype. From 22,733 genes, 454 transcripts were identified that were differentially expressed in *BRCA1/2* mutation carriers when compared with controls, pooling all ovarian and fallopian tube samples together. Of these, 299 genes were statistically significantly downregulated and 155 genes upregulated. Differentially expressed genes in *BRCA1/2* samples reported here might be involved in serous ovarian carcinogenesis and provide interesting targets for further studies.

INTRODUCTION

Germ-line mutations of the *BRCA1/2* genes are associated with an increased susceptibility for breast and ovarian cancer (Nelson et al., 2005). Risk-reducing salpingo-oophorectomy (RRSO) has been shown to minimize the risk of ovarian and fallopian tube cancer and also to substantially lower the risk of developing breast cancer among *BRCA1/2* mutation carriers (Kauff and Barakat, 2007). Data derived from RRSO studies have profoundly altered the insights of early pathogenesis of epithelial serous ovarian cancer, or the concept of distal fallopian tube epithelium, instead of ovarian surface epithelium (OSE), being the original site of early pathogenetic process in high grade serous ovarian cancer (Crum, 2009; Jarboe et al., 2009).

Epithelial ovarian cancer (EOC) is the most important cause of mortality among gynecological cancers (Kurman and Shih, 2011), and more than half of the EOCs are of high-grade serous (HGSC) type (Hannibal et al., 2012). Individualized therapy is warranted as EOC is a heterogeneous disease (McCluggage, 2011). Therefore, both identification of specific targets for treatment and improvement of early diagnostics remain a true challenge (Trinh et al., 2011).

Recent microarray technology and bioinformatics have been able to identify oncogenic cellular signaling pathways based on specific gene signatures in various cancers (Bild et al., 2006; Dressman et al., 2007; Gatza et al., 2010). Several

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microarray studies have been able to identify gene expression profiles associating with prognosis of HGSC (Konstantinopoulos et al., 2010; Kang et al., 2012; Yoshihara et al., 2012; Verhaak et al., 2013). Interestingly, gene expression array studies have been able to identify specific expression profiles also in *BRCA1*- and *BRCA2*-mutation positive ovarian cancers and these profiles are suggestive of a division of sporadic ovarian cancers in "*BRCA1*-like" and "*BRCA2*-like" tumors (Jazaeri et al., 2002). It has also been indicated that among ovarian cancer patients, *BRCA2* mutation carriers with no *BRCA1* deficiency have improved survival, illustrating differences in these subgroups (Yang et al., 2011). Moreover, a specific *BRCA*ness expression profile was found in a subgroup of sporadic ovarian cancer with other defects in homologous recombination and hence also responsive to the promising PARP-inhibitor therapy (Konstantinopoulos et al., 2010).

The purpose of this study was to combine the potential of prospective RRSO specimens derived from *BRCA1/2* mutation carriers possibly harboring early pathogenetic events in EOC development and microarray technology providing high throughput screening of genome-wide gene expression. Therefore we have prospectively collected RNA from both ovarian and fallopian tube samples from *BRCA1/2* mutation carriers scheduled for RRSO in order to identify differentially expressed genes potentially involved in early pathogenesis of EOC or as candidates of targeted therapy.

MATERIALS AND METHODS

The study was carried out in the University of Tampere and Tampere University Hospital (TAUH), Tampere, Finland. Local Ethics Committee approved the study protocol.

Tissue Samples

The prospective study cohort consisted of nine *BRCA1/2* mutation carriers undergoing RRSO and five control patients with no *BRCA1/2* mutation undergoing salpingo-oophorectomy for benign indications. The mean age of *BRCA1/2* mutation carriers at the time of RRSO surgery was 55 years. Seven out of nine (78%) mutation carriers had been diagnosed with breast cancer. Five control patients had no personal or family history of gynecological malignancy and were scheduled for adnexal surgery for benign reasons, for example, benign cyst unilaterally, in which case the tissue sample was taken from the macroscopically healthy-looking ovary or fallopian tube. The mean age of control patients at the time of surgery was 47 years.

Both fallopian tube and ovarian samples were collected from each *BRCA1/2* mutation carrier, resulting in eighteen mutation positive adnexal samples. Both fallopian tube and ovarian control samples were collected from one control patient while either an ovarian or a fallopian tube sample was available from four control patients, respectively, resulting in six adnexal control samples. The macroscopic and morphological findings were later confirmed by an experienced pathologist as part of routine diagnostics at the TAUH Department of Pathology. There were no occult tumors or premalignant lesions detected in *BRCA*-mutation positive RRSO samples. In addition, three samples of tumor tissue were obtained from patients with sporadic ovarian cancer undergoing surgery in TAUH. These HGSC ovarian cancer cases were selected for qRT-PCR validation assay based on their *BRCA*ness phenotype reflecting specific phenotypic characteristics and molecular defects of sporadic ovarian cancers shared with cancers linked to *BRCA* germ-line mutation (Tan et al., 2008).

Approximately, $0.5 \times 0.5 \times 0.5$ cm sized tissue samples of distal fallopian tube and ovarian surface were collected from the surgery specimens and were immediately snap-frozen with liquid nitrogen and stored in $-70°C$ for further analyses. Written informed consent was obtained from all study participants. The characteristics of study patients and samples are summarized in Table 1.

RNA Isolation

Approximately 30 mg of unprocessed tissue (i.e., containing both epithelium and underlying stromal tissue) was homogenized (TissueRupter, Qiagen, Venlo, The Netherlands) and total RNA was then automatically extracted using the QIAcube instrument with RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA concentration and quality was analyzed using the Agilent 2100 Bioanalyzer RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, CA) prior to microarray analysis. Samples with RNA concentration minimum of 100 ng/μl and RNA integrity number (RIN) value above eight were used for gene expression analysis. Finally, high quality RNA was available from nine *BRCA1/2*-mutation positive ovarian and eight *BRCA1/2*-mutation positive fallopian tube samples and from three control ovarian and three control fallopian tube samples (Table 1).

TABLE 1. Characteristics of Study and Control Patients

Patient No.	Mutation carrier[a]	*BRCA1* mutation	*BRCA2* mutation	Ovarian sample	Fallopian tube sample	Age at surgery (years)	History of breast cancer	Ovarian tumor tissue available	History of ovarian cancer
1	Yes	–	8327T>G	Yes	Yes[b]	70	Yes	No	No
2[c]	Yes	–	8327T>G	Yes	Yes	45	Yes	No	No
3	Yes	5214C>T	–	Yes	Yes	47	Yes	No	No
4[d]	Yes	–	9118-2A>G	Yes	Yes	48	No	No	No
5	Yes	–	7480C>T	Yes	Yes	51	Yes	No	No
6	Yes	nt3744	–	Yes	Yes	43	No	No	No
7	Yes	3640G>T	–	Yes	Yes	49	Yes	No	No
8	Yes	5214C>T	–	Yes	Yes	67	Yes	No	No
9	Yes	–	7480C>T	Yes	Yes	74	Yes	No	No
10	No	–	–	Yes	No	13	No	No	No
11	No	–	–	Yes	No	38	No	No	No
12	No	–	–	Yes	Yes	49	No	No	No
13	No	–	–	No	Yes	51	No	No	No
14	No	–	–	No	Yes	86	No	No	No
15	No	–	–	No	No	56	No	Yes	Yes
16	No	–	–	No	No	55	No	Yes	Yes
17	No	–	–	No	No	78	No	Yes	Yes

[a]Patients number 10–14 (controls) have not been tested for *BRCA1/2* mutation but have no personal or family history of ovarian or breast cancer and are therefore classified as mutation-negative. Patients 15–17 have distinct *BRCAness* phenotype, but have not been tested for *BRCA1/2* mutation. Macroscopically, normal ovarian and fallopian tube samples derived from patients 1–14 were included in the microarray and high-grade serous ovarian cancer samples derived from patients 15–17 were included only in the qRT-PCR validation assay.
[b]RNA sample with RIN value <8 and therefore omitted from microarray analysis.
[c]Patient number 2 is the daughter of patient number 1.
[d]Patient number 4 has undergone prophylactic mastectomy.

Gene Expression Array

One hundred nanogram of total RNA was used in analyses of gene expression. The Ambion WT Expression Kit was used to generate purified sense-strand cDNA (with incorporated 2′-Deoxyuridine, 5′-Triphosphate, dUTP). Fifteen ug of cRNA was used for single-stranded cDNA-synthesis (sscDNA). DNA was labeled by terminal deoxynucleotidyl transferase (TdT) with the Affymetrix® proprietary DNA Labeling Reagent that is covalently linked to biotin using the Affymetrix GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA). A total of 5.5 μg of sscDNA was fragmented and hybridized to the GeneChip array (Affymetrix Human Gene 1.0 ST Array) in cartridge format during 17-h incubation at 45°C. Immediately following hybridization, the array was washed and stained with streptavidin phycoerythrin conjugate using an automated protocol on the GeneChip® Fluidics Station 450, followed by scanning on a GeneChip® Scanner (Affymetrix Human Gene 1.0 ST Array).

Data Analysis

All ovarian and fallopian tube samples were patient matched. GeneChip® signal intensity data were processed with robust multiarray analysis using remapped gene annotations from the Brainarray Custom CDF files (HuGene10stv1_Hs_ENSG, v.14.1.0) (Gentleman et al., 2004). Additional gene information was extracted from EnsEMBL using BioMart and matching was done via EnsEMBL gene names. Analysis was implemented in R using the simpleaffy, limma, BioMart, and qvalue packages of the Bioconductor project (Storey and Tibshirani, 2003; Gentleman et al., 2004; Smyth, 2004; Durinck et al., 2005; Wilson and Miller, 2005). The significance of differential expression was assessed using the empirical Bayes moderated paired *t*-statistics followed by *P*-value adjustment with FDR (false discovery rate) approach (Smyth, 2004). Arrays were quality weighted before statistical testing (Ritchie et al., 2006). Genes with FDR corrected $P\text{-values} \leq 0.05$ were considered as differentially expressed. Quality of the data was assessed by checking quality values. Data were visualized by hierarchical clustering (HC) and principal component analysis (PCA). Data visualization was done in Matlab® (R2012b). Gene expression data have been loaded into the GEO website (http://www.ncbi.nlm.nih.gov/geo/).

Quantitative Real-Time PCR

The validation of microarray data was performed by qRT-PCR as previously described (Balacescu

TABLE 2. The 20 Most Upregulated Genes in *BRCA1/2* Ovarian and Fallopian Tube RRSO Samples

Gene id	Gene name	Gene description	Fold increase	*P*-value
ENSG00000201164	*U4*	U4 small nuclear RNA	3.21	0.011
ENSG00000001461	*NIPAL3*	NIPA-like domain containing 3 family member	2.65	0.027
ENSG00000103569	*AQP9*	Aquaporin 9	2.22	0.046
ENSG00000181690	*PLAG1*	Pleiomorphic adenoma gene 1	2.10	0.028
ENSG00000212829	*RPS26P3*	Ribosomal protein S26 pseudogene 3	2.09	0.046
ENSG00000221886	*C5orf54*	Chromosome 5 open reading frame 54	2.08	0.014
ENSG00000162753	*SLC9A11*	Solute carrier family 9, member 11	1.99	0.035
ENSG00000151882	*CCL28*	Chemokine (C-C motif) ligand 28	1.85	0.008
ENSG00000170421	*KRT8*	Keratin 8	1.84	0.049
ENSG00000197128	*ZNF772*	Zinc finger protein 772	1.77	0.007
ENSG00000167840	*ZNF232*	Zinc finger protein 232	1.73	0.005
ENSG00000143891	*GALM*	Galactose mutarotase	1.71	0.031
ENSG00000252690	*SCARNA15*	Small Cajal body-specific RNA 1	1.69	0.046
ENSG00000196981	*WDR5B*	WD repeat domain 5B	1.66	0.010
ENSG00000181631	*P2RY13*	Purinergic receptor P2Y, G-protein coupled, 13	1.66	0.015
ENSG00000144115	*THNSL2*	Threonine synthase-like 2 (S. Cerevisiae)	1.65	0.0104
ENSG00000170629	*DPY19L2P*	Dpy-19-like 2 pseudogene 2 (C. elegans)	1.65	0.015
ENSG00000199980	*Y_RNA*	Y RNA	1.63	0.046
ENSG00000162777	*DENND2D*	DENN/MADD, a signaling protein that has multiple functions	1.62	0.041
ENSG00000140563	*MCTP2*	Multiple C2 domains, transmembrane 2	1.57	0.046

RRSO, Risk-reducing salpingo-oophorectomy.

et al., 2014). The expression of six differently expressed genes was analyzed in the following 10 samples: four *BRCA1/2*-mutation positive, three control, and in three HGSC samples.

Total RNA of 385 ng was used in cDNA synthesis using DyNAmo cDNA Synthesis Kit (ThermoFisher Scientific, MA) according to the manufacturer's instructions. The primer-probe pairs for the selected genes were designed using Roche's Universal Probe Library Assay Design Center and *ACTB* was chosen as the reference gene. The detailed description of primer and probe sequences are available on request. Synthesized cDNA was diluted 1:50 prior to qPCR analyses, which were performed using LightCycler 480 Instrument II thermal cycler (Roche Applied Science, Pentzberg, Germany) with hydrolysis probes from Universal ProbeLibrary Set (Human) and Universal ProbeLibrary Human ACTB Gene Assay (Roche Applied Science). The specific thermocycling conditions are available on request. Negative controls (H_2O and negative reverse transcriptase control) were included in the assays. All samples were tested in triplicate and fell within the standard curve. Fold change in expression was calculated using the following formula [Gene of interest$_{Sample}$]/[Reference Gene$_{Sample}$])/([Gene of interest$_{Control}$]/[Reference Gene$_{Control}$].

Gene Ontology Enrichment Analysis

Gene ontology (GO) enrichment analysis was performed using the GO Slim ontology (Davey et al., 2011), GO human annotation files (ver. 6/11/2013), and experimentally validated interactions (EXP, IDA and IPI). Hypergeometric testing with Bonferroni correction was used to test the significance of the enrichment. The significance level threshold was set to 0.05. GO analysis was performed in Matlab® (R2012b) using the Bioinformatics Toolbox® functions to handle the GO structure.

RESULTS

Expression Array

From 22,733 genes, 454 transcripts were identified as differentially expressed in *BRCA1/2* mutation carriers when statistically compared with controls (all ovarian and fallopian tube samples were pooled together). Of these, 299 genes were significantly downregulated and 155 genes upregulated. The 20 most downregulated and upregulated genes are presented in Tables 2 and 3 and the complete list of the 454 differentially expressed genes or transcripts is available as supplemental data (Supporting Information Tables 1 and 2). When only *BRCA1/2*-mutation positive fallopian tube samples were compared with controls, altogether 148 transcripts were differentially expressed. Of these, 34 genes were upregulated (Supporting Information Table 3) and 114 were downregulated (Supporting Information Table 4). Twenty genes were coincidentally upregulated

TABLE 3. The 20 Most Downregulated Genes in *BRCA1/2* Ovarian and Fallopian Tube RRSO Samples

Gene id	Gene name	Gene description	Fold decrease	*P*-value
ENSG00000125740	*FOSB*	FBJ murine osteosarcoma viral oncogene homolog B	7.31	0.002
ENSG00000179388	*EGR3*	Early growth response 3	5.43	0.003
ENSG00000119508	*NR4A3*	Nuclear receptor subfamily 4, group A, member 3	5.00	0.003
ENSG00000142178	*SIK1*	Salt-inducible kinase1	4.93	0.001
ENSG00000081041	*CXCL2*	Chemokine (C-X-C motif) ligand 2	4.87	0.003
ENSG00000158859	*ADAMTS4*	ADAM metallopeptidase with thrombospondin type 1 motif	4.85	0.0008
ENSG00000184557	*SOCS3*	Suppressor of cytokine signaling 3	4.58	0.0004
ENSG00000153234	*NR4A2*	NR4A2 nuclear receptor subfamily 4, group A, member 2	4.50	0.012
ENSG00000128016	*ZFP36*	Zinc finger protein 36	4.14	0.003
ENSG00000144655	*CSRNP1*	Cysteine-serine-rich nuclear protein 1	4.09	0.001
ENSG00000142871	*CYR61*	Cysteine-rich, angiogenic inducer, 61	4.09	0.039
ENSG00000205364	*MT1M*	Metallothionein 1M	4.02	0.013
ENSG00000123358	*NR4A1*	A member of nuclear receptor family, involved in cell death	3.98	0.005
ENSG00000059804	*SLC2A3*	Solute carrier family 2	3.89	0.001
ENSG00000130844	*ZNF331*	Zinc finger protein 33	3.86	0.005
ENSG00000205362	*MT1A*	Metallothionein 1A	3.84	0.005
ENSG00000069849	*ATP1B3*	ATPase, Na+/K+ transporting, beta 3 polypeptide	3.83	0.003
ENSG00000113070	*HBEGF*	Heparin-binding EGF-like growth factor	3.72	0.003
ENSG00000136826	*KLF4*	Kruppel-like factor 4	3.60	0.0004
ENSG00000186594	*MIR22HG*	MIR22 host gene	3.55	0.005

RRSO, Risk-reducing salpingo-oophorectomy.

TABLE 4. Validation of Selected Differently Expressed Genes by Quantitative RT-PCR in *BRCA1/2* RRSO, Control[a] and High-Grade Serous Ovarian Carcinoma Samples

Gene	Rationale for selection	Expression in original array *BRCA1/2* RRSO vs. control ovarian and fallopian tube samples	Expression in *BRCA1/2* ovarian and fallopian tube samples ($n = 4$)[a]	Gene expression concordance *BRCA1/2* RRSO vs. control ovarian and fallopian tube samples	Expression in high-grade serous ovarian carcinoma ($n = 3$)[a]	Gene expression concordance that is. similar patterns of expression *BRCA1/2* RRSO vs. HGSC samples
KLF4[b]	Presence in four GO categories	3.4× downregulation	0.48	Yes	0.26	Yes
PLK[c]	Presence in three GO categories	2.0× downregulation	0.69	Yes	1.28	No
SIK1[d]	Presence in one GO category and foruth most downregulated gene in array	4.9× downreulation	0.16	Yes	0.07	Yes
TDP2[e]	Presence in two GO categories	1.5× upregulation	1.83	Yes	2.23	Yes
AQP9[f]	Third most upregulated gene in array	2.2× upregulation	6.32	Yes	2.12	Yes
EGR3[g]	Presence in one GO category and second most downregulated gene in array	5.4× downregulation	0.12	Yes	0.08	Yes

GO, gene ontology; RRSO, Risk-reducing salpingo-oophorectomy.
[a]Samples from normal ovarian and fallopian tube tissue from mutation-negative patients ($n = 4$); in quantitative RT-PCR analyses relative expression levels of selected genes in the control samples was set to 1.00 to which gene expression in *BRCA1/2* RRSO and serous ovarian cancer samples was then compared.
[b]Kruppel-like factor 4.
[c]Polo-like kinase 3.
[d]Salt-inducible kinase 1.
[e]Tyrosyl DNA phosphodiesterase-2.
[f]Aqua-glyceroporins 9.
[g]Early growth response 3.

TABLE 5. Enriched Biological Process Gene Ontology Categories for Differentially Expressed Genes in *BRCA1/2* Ovarian and Fallopian Tube RRSO Samples

GO Term	*P*-value	Counts (D.E./BG)	Definition
GO:0008219	3.32e-08	28/369	Cell death
GO:0009058	1.68e-07	58/1195	Biosynthetic process
GO:0006950	7.08e-07	33/550	Response to stress
GO:0048646	1.70e-05	9/77	Anatomical structure formation involved in morphogenesis
GO:0007165	2.36e-05	42/906	Signal transduction
GO:0034641	2.80e-05	55/1311	Cellular nitrogen compound metabolic process
GO:0006412	9.37e-05	8/76	Translation
GO:0040007	1.50e-04	9/99	Growth
GO:0008283	2.13e-04	17/283	Cell proliferation
Total D.E.	*443*		
Total Genes	*18181*		
Unique Gene Names	*16697*		

D.E., differentially expressed genes; D.E./BG, differentially expressed genes/background gene pool; GO, gene ontology; RRSO, Risk-reducing salpingo-oophorectomy.

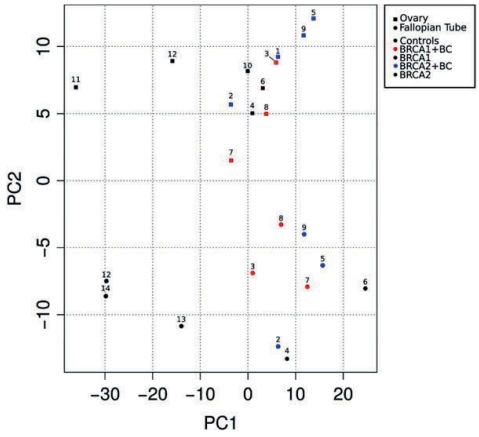

Figure 1. Scatterplot showing location of control, *BRCA1* and *BRCA2* ovarian and fallopian tube samples along the first two principal components (PC1 and PC2). Ovarian samples are represented by squares and fallopian tube samples by dots. Black color corresponds to control samples (i.e., mutation-negative samples), bright and dark red corresponds to samples from *BRCA1* mutation carriers with or without a personal history of breast cancer (BC), respectively. Cyan and blue color corresponds to samples from *BRCA2* mutation carriers with or without a personal history of breast cancer, respectively. Samples from study and control patients are numbered as in Table 1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and 80 downregulated when ovarian and fallopian tube samples were analyzed together or when the analyses were restricted only to fallopian tube samples. These overlapping differentially expressed genes are presented in Supporting Information Tables 5 and 6.

The global gene expression patterns were analyzed using PCA, which showed that ovarian and fallopian tube samples were separated from each other and that *BRCA1/2*-mutation positive samples mostly grouped together regardless of personal history of breast cancer (Fig. 1). To highlight the differences in gene expression, a supervised HC was performed on the set of differentially expressed genes. Expression profiles of *BRCA1/2*-mutation positive and control samples forming separate clusters are shown in Figure 2.

qRT-PCR

Six differentially expressed genes in microarray analysis were selected for validation by qRT-PCR. All the genes selected for qRT-PCR validation showed similar expression pattern as the original microarray where *BRCA1/2*-mutation positive RRSO samples were compared with control fallopian tube and ovarian samples. Five out of six genes showed comparable pattern of expression in *BRCA1/2*-mutation positive RRSO and HGSC samples. The summary of the qRT-PCR analyses is shown in Table 4.

GO Enrichment Analysis

GO enrichment analysis was performed to identify biological processes in which the identified differentially expressed genes are involved. Nine different GO terms showed a significant *P*-value and are summarized in Figure 3 and Table 5. Ten enriched biological processes in overlapping one hundred differentially expressed genes in ovarian and fallopian tube versus control samples and fallopian tube versus control samples were identified

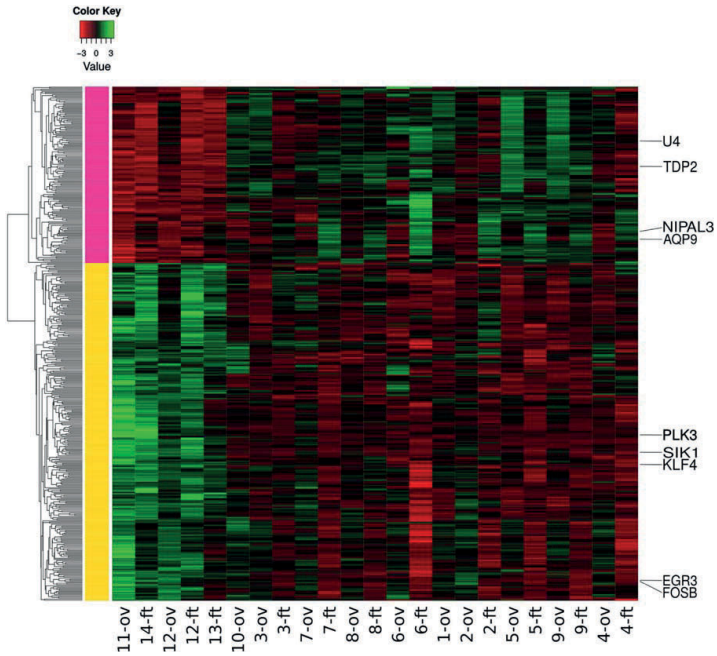

Figure 2. Heatmap of differentially expressed genes between *BRCA 1/2* ovarian and fallopian tube RRSO ($n = 17$; column sample numbers 1–9) and control samples ($n = 6$; column sample numbers 10–14), obtained from unsupervised hierarchical clustering. The color indicates the level of mRNA expression: red—higher level of expression; green—lower level of expression; black—no expression changes. Each row represents a gene. Fallopian tube samples are referred as *ft* and ovarian samples as *ov*. Samples from study and control patients are numbered as in Table 1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

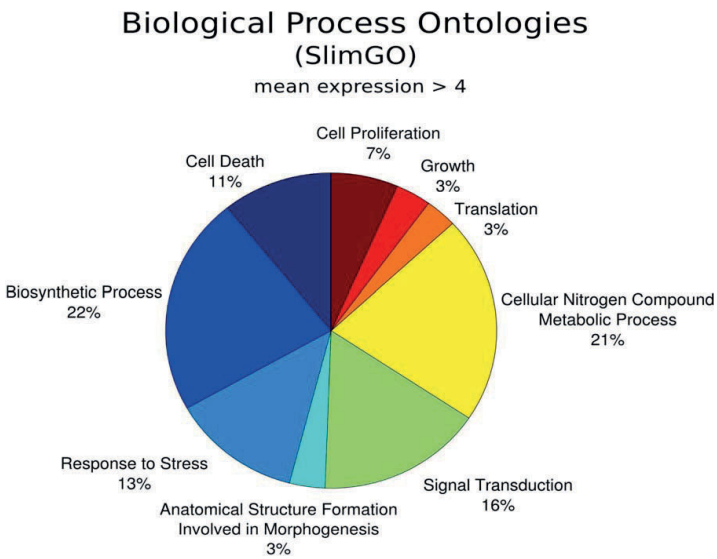

Figure 3. Pie chart showing relative proportions of enriched biological process GO categories for differentially expressed genes in *BRCA1/2* ovarian and fallopian tube RRSO samples. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and they are summarized in Supporting Information Figure 1.

DISCUSSION

We have studied the genome-wide gene expression levels in RRSO samples derived from *BRCA1/2* mutation carriers. Several reports on RRSO-derived specimens have been published, where histological and morphological analysis of paraffin-embedded archival samples has revealed interesting data on the site of initial ovarian carcinogenesis (Crum et al., 2007; Kurman and Shih, 2010).

There are only few studies on genome-wide gene expression levels in RRSO samples derived from *BRCA1/2* mutation carriers and data presented here provide a potentially valuable addition to the few previous studies (Tone et al., 2008; Press et al., 2010; George et al., 2011). Here, gene expression profiling by supervised HC or PCA showed distinct clustering of samples according to either tissue of origin or to *BRCA1/2*-mutation status. Our microarray analysis revealed a number of differentially expressed genes in *BRCA1/2* samples compared with controls. Downregulation of gene expression was more pronounced both in the amount (up to sevenfold decrease) and in the number of genes than upregulation (up to threefold increase). GO enrichment analysis was used to identify cellular and biological processes for differentially expressed genes in the array. GO enrichment analysis is widely recognized as the premier tool for the organization and functional annotation of extensive microarray data (Lovering et al., 2008).

The six following differentially expressed genes were chosen for qRT-PCR validation because of their presence in one or more GO category or top rank in the list of most up- or downregulated genes: *KLF4*, *PLK3*, *SIK1*, *TDP2*, *AQP9*, and *EGR3*. qRT-PCR results were in accordance with the microarray findings in all instances. In addition, gene expression of selected genes was compared between *BRCA1/2* RRSO and HGSC ovarian cancer samples by qRT-PCR. HGSC samples were selected for their positive *BRCA*ness phenotype, which has been associated with a specific gene expression profile predictive of response to chemotherapy (Konstantinopoulos et al., 2010). Moreover, specifically *BRCA1*-mutation seems to associate with defined gene expression profile in HGSC ovarian tumors distinct from *BRCA2*-positive or wild-type ovarian cancers (George et al., 2013). Despite of *BRCA2*-mutation positive RRSO samples included in our study, HGSC samples showing *BRCA*ness phenotype were regarded as the most suitable reference material available. In the qRT-PCR assay comparing *BRCA1/2* RRSO samples with HGSC samples, all but the *PLK3* gene showed comparable pattern of expression, suggesting that *KLF4*, *SIK1*, *TDP2*, *AQP9*, and *EGR3* may play a role in ovarian carcinogenesis or at least serve as interesting candidates for future studies. The discrepancy in *PLK3* expression may have several explanations. First, *PLK3* might not be important in ovarian cancer pathogenesis and differential expression of *PLK3* in *BRCA* RRSO samples in our microarray might be associated with some other features than inherited cancer predisposition of *BRCA* mutation carriers. Second, early and late events in cancer development might not be parallel and could at least partially explain the discordance in *PLK3* expression pattern in qRT-PCR validation assay, where *BRCA*-mutation positive macroscopically normal RRSO samples were compared with advanced stage HGSC samples. In fact, either overexpression or downregulation of PLK proteins occurs frequently in various cancer types (Pellegrino et al., 2010), and recent data have emphasized the relevance of *PLK3* in angiogenesis in tumor development (Xu et al., 2012).

Specifically, previous studies have shown that KLF4 is a key transcriptional regulator and is suggested to play a role in several malignancies, functioning as a potential tumor suppressor (Yori et al., 2011; Cui et al., 2013; Tetreault et al., 2013). There are only few reports on *KLF4* and EOC but one previous report showed downregulation of *KLF4* in ovarian cancer (Yoon and Roh, 2012). Our findings therefore are in line with previous data regarding *KLF4* and warrant further investigation on the role of *KLF4* in ovarian carcinogenesis. Here, *KLF4* was present in four GO categories, such as cellular nitrogen compound metabolic process, cell death, anatomical structure formation, and biosynthetic process. Moreover, there is evidence of *KLF4* regulating both JAK-STAT and NOTCH signaling pathways, alterations of which affect various cellular functions, such as inflammation, cell growth, and angiogenesis (Aittomäki and Pesu, 2014; Hale et al., 2014).

EGR3, the early growth response three, is a transcription factor regulating signal transduction, cell growth, and proliferation (Patwardhan et al., 1991). In our study, *EGR3* was the second most downregulated gene in the expression array and

was also downregulated in HGSC samples, suggesting that it is a potential tumor suppressor in ovarian cancer. *EGR3* was present in anatomical structure formation category in GO analysis. Interestingly, *EGR3* has not been previously associated with ovarian cancer, but its overexpression has been shown in nonrelapsing prostate cancer and decreased expression in gastric cancer has been associated with poor prognosis (Liao et al., 2013; Pio et al., 2013). Moreover, EGR3 has been shown to regulate the expression of SOCS3 (suppressor of cytokine signaling three, also downregulated in our array), also a negative regulator of JAK-STAT signaling pathway, aberrant function of which is possibly associated with tumor growth (Inagaki-Ohara et al., 2013). Taken together, its role in the EOC pathogenesis should be target of future investigation.

Another downregulated gene was *SIK1*, which is the fourth most downregulated gene in our expression array and also downregulated in HGSC samples. According to previous data, Salt-inducible kinases are a family of related serine-threonine kinases and involved, for example, in liver glucose homeostasis, steroidogenesis, and adipogenesis (Yong Kim et al., 2013). In our GO analysis, *SIK1* was present in signal transduction category in accordance with its multiple roles. In terms of carcinogenesis, *SIK1* has been shown to suppress metastasis in a TP53-dependent manner (Cheng et al., 2009) and to regulate E-cadherin expression affecting intercellular junction stability (Eneling et al., 2012). To our knowledge *SIK1* has not been previously linked to ovarian cancer. In the light of our microarray analysis and previously published data, *SIK1* provides an interesting target for future ovarian cancer studies.

Two upregulated genes validated by qRT-PCR were *AQP9* and *TDP2*. *AQP9* belongs to aquaporins, which are integral transmembrane water channel proteins involved in transcellular water movement, transport of fluid, and cell migration (Ribatti et al., 2014). *AQP9* has been associated with several cancers, such as lung, prostate, brain, and ovarian cancer (Yang et al., 2011; Fossdal et al., 2012; Hwang et al., 2012; Wang et al., 2014). According to previous data, aquaporin expression in tumor cells and vessels facilitates tumor growth and spread, suggesting aquaporin inhibition as a novel antitumor therapy (Verkman et al., 2008). *AQP9* was the third most upregulated gene in array and also upregulated in ovarian cancer tissue, suggesting that *AQP9* serves as promising target for further investigation.

TDP2 is a phosphodiesterase that prevents DNA breaks induced by aberrant topoisomerase II activity (Gao et al., 2014). It has not been previously associated with ovarian cancer, but it has been shown to act as an oncogenic agent, and some data also suggest it has a role in chemoresistance (Li et al., 2011). Here, *TDP2* was upregulated in *BRCA1/2* mutation carriers and ovarian cancer tissue. It was involved in two GO categories: response to stress and cellular nitrogen compound metabolic process. Our findings suggest that *TDP2* may have a role in EOC pathogenesis, warranting further investigations. It could be speculated that impaired *BRCA1/2* function in DNA repair by homologous recombination (Li and Heyer, 2008) could also lead to upregulation of *TDP2* and that it therefore serves as an interesting target for further studies, especially in regard to EOC treatment.

Our findings are mostly in accordance with previous data on gene expression in *BRCA*-mutation positive RRSO specimens despite certain distinct differences in study materials (Tone et al., 2008; Press et al., 2010; George et al., 2011). Previous reports have mainly focused on *BRCA1*-mutation samples (Press et al., 2010; George et al., 2011) and exclusively on fallopian tube epithelium selected by laser capture microdissection (Tone et al., 2008; Press et al., 2010; George et al., 2011). Regardless of the current understanding of fallopian tube as origin of progenitor cells of HGSC, we aimed at analyzing gene expression from both ovarian and fallopian tube samples. This was partially based on recent in vivo animal data suggesting that ovaries may, in fact, interact with fallopian tube in the invasive process by providing a favorable hormonal environment for development of advanced disease (Perets et al., 2013). Our study protocol did not include laser capture microdissection, partly due to lack of premalignant lesions in RRSO specimens. In addition, we did not want to rule out the effect of possible interaction between stromal and epithelial tissue. In fact, ovarian epithelial cell interplay with the immune system with regard to folliculogenesis has been established long ago (Bukovský et al., 1995), and such an interplay is possibly present also in HGSC (Martins Filho et al., 2014). Regardless of these differences, many of the differentially expressed genes reported here have also been implicated previously, like aquaporins, Kruppel-like transcription factors (KLFs), interleukins, and suppressor of cytokine signaling genes (e.g., SOCS3), emphasizing the possible relevance of the differentially expressed genes and involved cellular pathways

revealed by microarray technology (Tone et al., 2008; Press et al., 2010; George et al., 2011).

When gene expression was studied separately only in *BRCA1/2*-mutation positive fallopian tubes in comparison with controls, *KLF4*, *SIK1*, and *AQP9* were all differentially expressed and *EGR3* was at the limit of significant downregulation ($P = 0.05$). In the light of the fallopian tube theory in EOC pathogenesis, this subgroup analysis may emphasize the significance of differential gene expression in the case of these genes. *TDP2*, however, was not significantly expressed in this subgroup analysis, but the quite small sample size has to be taken into account. Small, but possibly biologically relevant differences in gene expression can escape notice, a fact that applies also for the whole array study even with all ovarian and fallopian tube samples analyzed together.

There are limitations to our study. As already briefly discussed above, the number of mutation positive and control samples was limited, but it is important to note that the array used in the study determined the total number of samples studied. In addition, due to rarity of germ-line *BRCA1/2* mutations (Edlich et al., 2005), collection of considerable number of patients is challenging. However, the aim of this study was to screen for differences in global gene expression in a well-defined patient population of *BRCA1/2*-mutation carriers serving as a starting point for more detailed future studies. Some of the differences in gene expression can be explained by *BRCA1/2*-mutation carriers being older than the control patients. In addition, some of the mutation carriers were of rather high age at time of the RRSO surgery and they might not serve as ideal study patients of predisposition for ovarian cancer at young age. However, as *BRCA* mutations are associated with an increased life-time risk of ovarian cancer and these patients also had a personal history of breast cancer, indicative of actualized personal cancer predisposition, they were included in the study. In addition, archival paraffin-embedded RRSO samples of *BRCA1/2*-mutation positive patients in the present study have been previously studied for TP53 nuclear immunoreactivity, which was shown to be significantly higher compared with control adnexal samples, further justifying the inclusion of all *BRCA*-mutation positive samples in the present study (Staff et al., 2014).

In conclusion, we present microarray data of differentially expressed genes in *BRCA1/2*-mutation positive RRSO samples. Selected genes were validated by qRT-PCR showing comparable expression patterns between *BRCA1/2*-mutation positive RRSO and HGSC samples, suggesting that microarray analysis was able to reveal potential candidate genes for future research on EOC pathogenesis and targeted therapy.

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REFERENCES

Aittomäki S, Pesu M. 2014. Therapeutic targeting of the Jak/STAT pathway. Basic Clin Pharmacol Toxicol 114:18–23.

Balacescu O, Balacescu L, Tudoran O, Todor N, Rus M, Buiga R, Susman S, Fetica B, Pop L, Maja L, Visan S, Ordeanu C, Berindan-Neagoe I, Nagy V. 2014. Gene expression profiling reveals activation of the FA/BRCA pathway in advanced squamous cervical cancer with intrinsic resistance and therapy failure. BMC Cancer 14:246.

Bild AH, Yao G, Chang JT, Wang Q, Potti A, Chasse D, Joshi MB, Harpole D, Lancaster JM,Berchuck A, Olson JA, Jr., Marks JR, Dressman HK, West M, Nevins JR. 2006. Oncogenic pathway signatures in human cancers as a guide to targeted therapies. Nature 439:353–357.

Bukovský A, Keenan JA, Caudle MR, Wimalasena J, Upadhyaya NB, Van Meter SE. 1995. Immunohistochemical studies of the adult human ovary: possible contribution of immune and epithelial factors to folliculogenesis. Am J Reprod Immunol 33: 323–340.

Cheng H, Liu P, Wang ZC, Zou L, Santiago S, Garbitt V, Gjoerup OV, Iglehart JD, Miron A, Richardson AL, Hahn WC, Zhao JJ. 2009. SIK1 couples LKB1 to p53-dependent anoikis and suppresses metastasis. Sci Signal 2:ra35.

Crum C. 2009. Intercepting pelvic cancer in the distal fallopian tube: theories and realities. Mol Oncol 3:165–170.

Crum CP, Drapkin R, Kindelberger D, Medeiros F, Miron A, Lee Y. 2007. Lessons from BRCA: the tubal fimbria emerges as an origin for pelvic serous cancer. Clin Med Res 5:35–44.

Cui J, Shi M, Quan M, Xie K. 2013. Regulation of EMT by KLF4 in gastrointestinal cancer. Curr Cancer Drug Targets 13: 986–995.

Davey NE, Haslam NJ, Shields DC, Edwards RJ. 2011. SLiMSearch 2.0: biological context for short linear motifs in proteins. Nucleic Acids Res 39(Web Server issue) W56–W60.

Dressman HK, Berchuck A, Chan G, Zhai J, Bild A, Sayer R, Cragun J, Clarke J, Whitaker RS, Li L, Gray J, Marks J, Ginsburg GS, Potti A, West M, Nevins JR, Lancaster JM. 2007. An integrated genomic-based approach to individualized treatment of patients with advanced-stage ovarian cancer. J Clin Oncol 25:517–525.

Durinck S, Moreau Y, Kasprzyk A, Davis S, De Moor B, Brazma A, Huber W. 2005. BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. Bioinformatics 21:3439–3440.

Edlich RF, Winters KL, Lin KY. 2005. Breast cancer and ovarian cancer genetics. J Long Term Eff Med Implants 15: 533–545.

Eneling K, Brion L, Pinto V, Pinho MJ, Sznajder JI, Mochizuki N, Emoto K, Soares-da-Silva P, Bertorello AM. 2012. Salt-inducible kinase 1 regulates E-cadherin expression and intercellular junction stability. FASEB J 26:3230–3239.

Fossdal G, Vik-Mo EO, Sandberg C, Varghese M, Kaarbø M, Telmo E, Langmoen IA, Murrell W. 2012. AQP9 and brain tumour stem cells. Sci World J 2012:915176.

Gao R, Schellenberg MJ, Huang SY, Abdelmalak M, Marchand C, Nitiss KC, Nitiss JL, Williams RS, Pommier Y. 2014. Proteolytic Degradation of Topoisomerase II (Top2) Enables the

Processing of Top2-DNA and -RNA Covalent Complexes by Tyrosyl-DNA-phosphodiesterase 2 (TDP2). J Biol Chem 289: 17960–17969.

Gatza ML, Lucas JE, Barry WT, Kim JW, Wang Q, Crawford MD, Datto MB, Kelley M, Mathey Prevot B, Potti A, Nevins JR. 2010. A pathway-based classification of human breast cancer. Proc Natl Acad Sci USA 107:6994–6999.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J. 2004. Bioconductor: Open software development for computational biology and bioinformatics. Genome Biol 5:R80.

George J, Alsop K, Etemadmoghadam D, Hondow H, Mikeska T, Dobrovic A, deFazio A; Australian Ovarian Cancer Study Group, Smyth GK, Levine DA, Mitchell G, Bowtell DD. 2013. Nonequivalent gene expression and copy number alterations in high-grade serous ovarian cancers with BRCA1 and BRCA2 mutations. Clin Cancer Res 19:3474–3484.

George SH, Greenaway J, Milea A, Clary V, Shaw S, Sharma M, Virtanen C, Shaw PA. 2011. Identification of abrogated pathways in fallopian tube epithelium from BRCA1 mutation carriers. J Pathol 225:106–117.

Hale AT, Tian H, Anih E, Recio FO 3rd, Shatat MA, Johnson T, Liao X, Ramirez-Bergeron DL, Proweller A, Ishikawa M, Hamik A. 2014. Endothelial Kruppel-like factor 4 regulates angiogenesis and the Notch signaling pathway. J Biol Chem 289:12016–120128.

Hannibal CG, Vang R, Junge J, Kjaerbye-Thygesen A, Kurman RJ, Kjaer SK. 2012. A binary histologic grading system for ovarian serous carcinoma is an independent prognostic factor: a population-based study of 4317 women diagnosed in Denmark 1978–2006. Gynecol Oncol 125:655–660.

Hwang I, Jung SI, Hwang EC, Song SH, Lee HS, Kim SO, Kang TW, Kwon D, Park K. 2012. Expression and localization of aquaporins in benign prostate hyperplasia and prostate cancer. Chonnam Med J 48:174–178.

Inagaki-Ohara K, Kondo T, Ito M, Yoshimura A. 2013. SOCS, inflammation, and cancer. JAKSTAT 2:e24053.

Jarboe EA, Folkins AK, Drapkin R, Ince TA, Agoston ES, Crum CP. 2009. Tubal and ovarian pathways to pelvic epithelial cancer: A pathological perspective. Histopathology 55: 619.

Jazaeri AA, Yee CJ, Sotiriou C, Brantley KR, Boyd J, Liu ET. 2002. Gene expression profiles of BRCA1-linked, BRCA2-linked, and sporadic ovarian cancers. J Natl Cancer Inst 94:990–1000.

Kang J, D'Andrea AD, Kozono D. 2012. A DNA repair pathway-focused score for prediction of outcomes in ovarian cancer treated with platinum-based chemotherapy. J Natl Cancer Inst 104:670–681.

Kauff N, Barakat R. 2007. Risk-reducing salpingo-oophorectomy in patients with germline mutations in BRCA1 or BRCA2. J Clin Oncol 25:2921–2927.

Konstantinopoulos PA, Spentzos D, Karlan BY, Taniguchi T, Fountzilas E, Francoeur N, Levine DA, Cannistra SA. 2010. Gene expression profile of *BRCAness* that correlates with responsiveness to chemotherapy and with outcome in patients with epithelial ovarian cancer. J Clin Oncol 28:3555–3561.

Kurman RJ, Shih IeM. 2010. The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory. Am J Surg Pathol 34:433–443.

Kurman RJ, Shih IeM. 2011. Molecular pathogenesis and extraovarian origin of epithelial ovarian cancer–shifting the paradigm. Hum Pathol 42:918–931.

Li C, Sun SY, Khuri FR, Li R. 2011. Pleiotropic functions of EAPII/TTRAP/TDP2: Cancer development, chemoresistance and beyond. Cell Cycle 10:3274–3283.

Li X, Heyer WD. 2008. Homologous recombination in DNA repair and DNA damage tolerance. Cell Res 18:99–113.

Liao F, Ji MY, Shen L, Qiu S, Guo XF, Dong WG. 2013. Decreased EGR3 expression is related to poor prognosis in patients with gastric cancer. J Mol Histol 44:463–468.

Lovering RC, Camon EB, Blake JA, Diehl AD. 2008. Access to immunology through the Gene Ontology. Immunology 125: 154–160.

Martins Filho A, Jammal MP, Nomelini RS, Murta EF. 2014. The immune response in malignant ovarian neoplasms. Eur J Gynaecol Oncol 35:487–491.

McCluggage WG. 2011. Morphological subtypes of ovarian carcinoma: a review with emphasis on new developments and pathogenesis. Pathology 43:420–432.

Nelson HD, Huffman LH, Fu R, Harris EL; U.S. Preventive Services Task Force. 2005. Genetic risk assessment and BRCA mutation testing for breast and ovarian cancer susceptibility: systematic evidence review for the U.S. Preventive Services Task Force. Ann Intern Med 143:362–379.

Patwardhan S, Gashler A, Siegel MG, Chang LC, Joseph LJ, Shows TB, Le Beau MM, Sukhatme VP. 1991. EGR3, a novel member of the Egr family of genes encoding immediate-early transcription factors. Oncogene 6:917–928.

Pellegrino R, Calvisi DF, Ladu S, Ehemann V, Staniscia T, Evert M, Dombrowski F, Schirmacher P, Longerich T. 2010. Oncogenic and tumor suppressive roles of polo-like kinases in human hepatocellular carcinoma. Hepatology 51:857–868.

Perets R, Wyant GA, Muto KW, Bijron JG, Poole BB, Chin KT, Chen JY, Ohman AW, Stepule CD, Kwak S, Karst AM, Hirsch MS, Setlur SR, Crum CP, Dinulescu DM, Drapkin R. 2013. Transformation of the fallopian tube secretory epithelium leads to high-grade serous ovarian cancer in Brca; Tp53; Pten models. Cancer Cell 24:751–765.

Pio R, Jia Z, Baron VT, Mercola D. 2013. Early growth response 3 (Egr3) is highly over-expressed in non-relapsing prostate cancer but not in relapsing prostate cancer. PLoS One 8:e54096.

Press JZ, Wurz K, Norquist BM, Lee MK, Pennil C, Garcia R, Welcsh P, Goff BA, Swisher EM. 2010. Identification of a preneoplastic gene expression profile in tubal epithelium of BRCA1 mutation carriers. Neoplasia 12:993–1002.

Ribatti D, Ranieri G, Annese T, Nico B. 2014. Aquaporins in cancer. Biochim Biophys Acta 1840:1550–1553.

Ritchie ME, Diyagama D, Neilson J, van Laar R, Dobrovic A, Holloway A, Smyth GK. 2006. Empirical array quality weights in the analysis of microarray data. BMC Bioinform 7:261.

Smyth GK. 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3:Article3.

Staff S, Tolonen T, Laasanen SL, Mecklin JP, Isola J, Mäenpää J. 2014. Quantitative analysis of γ H2AX and p53 nuclear expression levels in ovarian and fallopian tube epithelium from risk-reducing salpingo-oophorectomies in BRCA1 and BRCA2 mutation carriers. Int J Gynecol Pathol 33:309–316.

Storey JD, Tibshirani R. 2003. Statistical significance for genome-wide studies. Proc Natl Acad Sci USA 100:9440–9445.

Tan DS, Rothermundt C, Thomas K, Bancroft E, Eeles R, Shanley S, Ardern-Jones A, Norman A, Kaye SB, Gore ME. 2008. "BRCAness" syndrome in ovarian cancer: a case-control study describing the clinical features and outcome of patients with epithelial ovarian cancer associated with BRCA1 and BRCA2 mutations. J Clin Oncol 26:5530–5536.

Tetreault MP, Yang Y, Katz JP. 2013. Krüppel-like factors in cancer. Nat Rev Cancer 13:701–713.

Tone AA, Begley H, Sharma M, Murphy J, Rosen B, Brown TJ, Shaw PA. 2008. Gene expression profiles of luteal phase fallopian tube epithelium from BRCA mutation carriers resemble high-grade serous carcinoma. Clin Cancer Res. 14: 4067–4078.

Trinh XB, Tjalma WA, Dirix LY, Vermeulen PB, Peeters DJ, Bachvarov D, Plante M, Berns EM, Helleman J, Van Laere SJ, van Dam PA. 2011. Microarray-based oncogenic pathway profiling in advanced serous papillary ovarian carcinoma. PLoS One 6: e22469.

Verhaak RG, Tamayo P, Yang JY, Hubbard D, Zhang H, Creighton CJ, Fereday S, Lawrence M, Carter SL, Mermel CH, Kostic AD, Etemadmoghadam D, Saksena G, Cibulskis K, Duraisamy S, Levanon K, Sougnez C, Tsherniak A, Gomez S, Onofrio R, Gabriel S, Chin L, Zhang N, Spellman PT, Zhang Y, Akbani R, Hoadley KA, Kahn A, Köbel M, Huntsman D, Soslow RA, Defazio A, Birrer MJ, Gray JW, Weinstein JN, Bowtell DD, Drapkin R, Mesirov JP, Getz G, Levine DA, Meyerson M. 2013. Prognostically relevant gene signatures of high-grade serous ovarian carcinoma. J Clin Invest 123: 517–525.

Verkman AS, Hara-Chikuma M, Papadopoulos MC. 2008. Aquaporins—New players in cancer biology. J Mol Med (Berl) 86:523–529.

Wang Y, Yin JY, Li XP, Chen J, Qian CY, Zheng Y, Fu YL, Chen ZY, Zhou HH, Liu ZQ. 2014. The association of transporter genes polymorphisms and lung cancer chemotherapy response. PLoS One 9:e91967.

Wilson CL, Miller CJ. 2005. Simpleaffy: A BioConductor package for Affymetrix Quality Control and data analysis. Bioinformatics 21:3683–3685.

Xu D, Wang Q, Jiang Y, Zhang Y, Vega-Saenzdemiera E, Osman I, Dai W. 2012. Roles of Polo like kinase 3 in suppressing tumor angiogenesis. Exp Hematol Oncol 1:5.

Yang D, Khan S, Sun Y, Hess K, Shmulevich I, Sood AK, Zhang W. 2011. Association of BRCA1 and BRCA2 mutations with survival, chemotherapy sensitivity, and gene mutator phenotype in patients with ovarian cancer. JAMA 306:1557–1565.

Yang JH, Yan CX, Chen XJ, Zhu YS. 2011. Expression of aquaglyceroporins in epithelial ovarian tumours and their clinical significance. J Int Med Res 39:702–711.

Yong Kim S, Jeong S, Chah KH, Jung E, Baek KH, Kim ST, Shim JH, Chun E, Lee KY. 2013. Salt-inducible kinases 1 and 3 negatively regulate Toll-like receptor 4-mediated signal. Mol Endocrinol 27:1958–1968.

Yoon O, Roh J. 2012. Downregulation of KLF4 and the Bcl-2/Bax ratio in advanced epithelial ovarian cancer. Oncol Lett 4:1033–1036.

Yori JL, Seachrist DD, Johnson E, Lozada KL, Abdul-Karim FW, Chodosh LA, Schiemann WP, Keri RA. 2011. Krüppel-like factor 4 inhibits tumorigenic progression and metastasis in a mouse model of breast cancer. Neoplasia 13:601–610.

Yoshihara K, Tsunoda T, Shigemizu D, Fujiwara H, Hatae M, Fujiwara H, Masuzaki H, Katabuchi H, Kawakami Y, Okamoto A, Nogawa T, Matsumura N, Udagawa Y, Saito T, Itamochi H, Takano M, Miyagi E, Sudo T, Ushijima K, Iwase H, Seki H, Terao Y, Enomoto T, Mikami M, Akazawa K, Tsuda H, Moriya T, Tajima A, Inoue I, Tanaka Warnings484 Dot is in crm style. 2012. High-risk ovarian cancer based on 126-gene expression signature is uniquely characterized by downregulation of antigen presentation pathway. Clin Cancer Res 18:1374–1385.

PUBLICATION

II

Assessment of PARP protein expression in epithelial ovarian cancer by ELISA pharmacodynamic assay and immunohistochemistry

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ORIGINAL ARTICLE

Assessment of PARP protein expression in epithelial ovarian cancer by ELISA pharmacodynamic assay and immunohistochemistry

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Abstract Targeting Poly (ADP-ribose) polymerase 1 (PARP-1) involved in base excision repair (BER) has been shown to be a clinically effective treatment strategy in epithelial ovarian cancer (EOC) defective in homologous recombination (HR). The aim of this study was to evaluate fresh EOC tumor tissue in regard to PAR (Poly (ADP-ribose)) concentration as a surrogate marker for PARP activity and PARP protein expression in archival samples by immunohistochemistry (IHC). The prospective study cohort consisted of 57 fresh tumor samples derived from patients undergoing primary ($n = 38$) or interval debulking surgery ($n = 19$) for EOC and parallel archival paraffin-embedded tumor samples. PARP activity in fresh frozen tumor tissue was assessed by an enzymatic chemiluminescence assay and PARP protein expression in paraffin-embedded tumor tissue by IHC. No correlation was detected between PARP enzyme activity and PARP staining by IHC ($p = 0.82$). High PARP activity was associated with platinum sensitivity both in the entire study cohort ($p = 0.022$) and in the high-grade subgroup ($p = 0.017$). High PARP activity was also associated with improved progression-free survival (PFS) (32 vs 14 months, log-rank $p = 0.009$). However, PARP immunostaining pattern was not predictive of patient survival. In conclusion, we present a novel finding of high PARP activity associated with platinum sensitivity and improved PFS in EOC. There was no association between PARP IHC and pharmacodynamic assay, and the correlation of PARP IHC with clinico-pathological characteristics and patient survival was poor. Pharmacodynamic assay rather than IHC seems to reflect better biologically significant PARP.

Keywords PARP · Epithelial ovarian cancer · BRCA · Platinum sensitivity · IHC

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Introduction

Epithelial ovarian cancer (EOC) is a heterogeneous disease with distinct epidemiological, phenotypical, and molecular subtypes including high-grade serous carcinoma (HGSC), low-grade serous, mucinous, endometrioid, and clear cell carcinoma [1]. Genetically complex and unstable HGSC is the most prevalent histotype and responsible for the highest mortality associated with EOC [1].

Cytoreductive surgery followed by platinum-taxane based combination chemotherapy is the cornerstone of the treatment of EOC [2]. Although initial response rates to chemotherapy are high, majority of patients will relapse, leading ultimately to the development of chemoresistance [3]. Therefore, unraveling the molecular mechanisms leading to platinum resistance constitutes a true challenge in current EOC research.

There is emerging evidence that sufficiently functional DNA repair and intact homologous recombination are major determinants of platinum resistance [4, 5]. The detailed analysis of EOC

by TCGA network has revealed a homologous recombination defect (HRd) in approximately 50 % of HGSC cases [6]. HRd not only results from germ-line or somatic mutations or epigenetic silencing of *BRCA1/2* genes but also from defects in various other genes such as *EMSY*, *RAD51C*, *ATR*, *ATM*, *PTEN*, and genes in Fanconi anemia (FA) pathway [7]. Compatible with the possible association between HR status and response to platinum therapy, *BRCA1/2* mutation carriers respond better to platinum-based chemotherapy [8] and have an improved 5-year overall survival as compared to sporadic cases [9, 10]. *BRCA*ness profile has been suggested to identify a specific phenotype of sporadic high-grade OC with responsiveness to platinum therapy and improved outcome comparable to *BRCA1/2* germ-line mutation carriers [11].

In the setting of defective HR, inhibition of a second DNA repair pathway such as base excision repair (BER), results in synthetic lethality [12]. Poly (ADP-ribose) polymerase 1 (PARP-1) has been identified as a key enzyme involved in BER [13, 14]. The observation of synthetic lethality was followed by development of PARP inhibitors [12, 15], which were originally designed to potentiate the tumor-killing activity of ionizing radiation and genotoxic agents, such as temozolomide (TMZ) and topotecan [16]. PARP inhibition has been shown to be an effective treatment strategy in various malignant tumor types, especially EOC, derived from *BRCA1/2* germ-line mutation carriers [17, 18]. Recently olaparib, a potent PARP inhibitor, was found to provide a significant progression-free survival (PFS) benefit over placebo as a maintenance therapy in platinum-sensitive recurrent *BRCA1/2*-mutation positive HGSC [19, 20].

In addition to *BRCA1/2* mutations, deficiency in other HR genes may also cause sensitivity to PARP inhibitors [6, 21]. In fact, the clinical efficacy of olaparib, albeit to a lesser degree, was seen also in wild-type (i.e., non-*BRCA1/2* mutation) platinum-sensitive OC [20]. The sensitivity of HRd cells to PARP inhibitors exceeds that of any other class of compounds by at least one order of magnitude, suggesting that other functions of PARP may also underlie the strong synthetic lethal interaction observed [22]. Identification of aberrant DNA repair pathways in other histological subtypes of OC and combination of PARP inhibitors with other biologic agents will likely increase the number of patients benefiting from PARP inhibitors [23]. In fact, a recent study suggests HRd to be a common event not only in serous but also in non-serous OC subtypes highlighting the potential activity of PARP inhibition beyond *BRCA*ness and HGSC subtype [24].

To our knowledge, no data regarding PARP activity in fresh EOC tumor tissue are available at present. In clinical OC, there are previous data regarding *PARP1* mRNA expression by microarray technology [25, 26] and PARP protein content by immunohistochemistry [26–28]. However, the available data concerning clinical correlations of PARP IHC are contradictory [25, 29]. Therefore, the aim of this study was to analyze both PAR concentration as an indirect measurement of PARP activity in fresh EOC tumor tissue by a pharmacodynamic assay [30] and PARP protein expression from parallel paraffin-embedded archival tumor samples by IHC. Both PARP activity and PARP immunostaining pattern were correlated with clinico-pathological characteristics and response to chemotherapy.

Patients and methods

The study was carried out at the University of Tampere and Tampere University Hospital (TAUH), Tampere, Finland. The study protocol was approved by the Ethics Committee of TAUH (Identification code ETL-R11137, September 13, 2011).

Study cohort and tissue samples

The prospective study cohort consisted of 100 consecutive women providing an informed consent and scheduled either for primary or interval debulking surgery for suspected or histologically verified ovarian cancer at TAUH in 2012–2013. Fresh samples from tumor tissue were collected from each enrolled patient at surgery. Two samples sized $0.5 \times 0.5 \times 0.5$ cm were chosen at the operation room from the surgery specimens and were immediately snap-frozen with liquid nitrogen and stored in −70 °C for further analyses. In the cases of interval debulking surgery, fresh tumor samples were taken from the macroscopically visible residual tumor. The morphological and histological findings from the corresponding archival surgical tumor specimens were assessed by experienced pathologists as part of routine diagnostics at the Department of Pathology of TAUH. Only samples from patients with histologically verified primary epithelial ovarian malignancy were included in the final study cohort for further analyses of PAR concentration. Thus, the final study cohort consisted of 57 women with fresh samples from primary epithelial ovarian cancer.

Clinical characteristics

Clinical and pathological data, including age at diagnosis, date of primary or interval surgery, FIGO stage, grade, and histological tumor type were collected from the patient records. Clinical data included patient's height, weight, history of breast cancer, systemic hormone replacement therapy (HRT), and smoking. Stage was confirmed by a thorough review of the operative and pathology reports. In addition, data regarding chemotherapy were obtained as follows: number of treatment cycles, platinum sensitivity defined as no recurrence within 12 months after the completion of first-line platinum-based chemotherapy, use of neoadjuvant chemotherapy (NACT), and CA12-5 level before, during, and after chemotherapy. Follow-up data were collected from

medical records by documenting the time of recurrence or the last follow-up visit or death, respectively.

The mean age of the patients was 66 years (SD 9.3). Most tumors were high-grade (70 %) with serous (84 %) histology. The median follow-up time was 31 months (range 2–50 months). The main clinico-pathological characteristics of study patients are summarized in Table 1.

Assessment of PAR concentration

For the measurement of PAR (Poly (ADP-ribose)) concentration in tumor tissue, the HT PARP in vivo Pharmacodynamic Assay II by Trevigen (Gaithersburg, MD, USA) was used. In response to DNA damage, as a result of its enzymatic activity, PARP-1, the main isoform of PARP family, catalyzes the NAD^+-dependent addition of PAR onto itself and adjacent nuclear proteins [25]. Therefore, the measurement of PAR concentration can be regarded as an indirect measurement of PARP-1 activity.

The pharmacodynamic assay was run according to the instructions by the manufacturer. Briefly, approximately 10 mg of frozen tissue sample was minced in lysis buffer, homogenized with vortex, and sonicated three times in 10 s cycles with 23 % amplitude (Sonics, Vibra-Cell, Newtown, CT, USA). Sodium dodecyl sulfate (SDS) was added to the lysates to the final concentration of 1.0 %, and samples were incubated at 100 °C for 5 min. After centrifugation, supernatants were collected and protein concentration was measured with BCA protein assay kit (Thermo Fisher Scientific, MA, USA). Equal amounts of protein lysates were analyzed in duplicates with ELISA assay according to the manufacturer's instructions, and PAR concentrations were quantified based on chemiluminescent signal measurement (Luminoskan Ascent, Thermo Fisher Scientific, MA, USA). Initially, 3 and 10 μg protein lysates were tested in parallel, and values with 3 μg of the total protein were found to allocate better to the PAR standard curve (detection range 10–1000 pg/ml). Samples were run in duplicates and averaged to achieve the final concentration. In addition, commercial protein lysate controls with predetermined PAR concentrations were included in the assay.

Table 1 Characteristics of study patients

Characteristic	
Patients in study, *n*	57
Age at surgery, years (SD)	66 (9.3)
BMI, mean (SD)	26.8 (6.2)
Median follow-up, months (range)	31 (2–50)
Ca 125 before treatment, median (range)	490.5 (25–4728)
FIGO stage, *n* (%)	
Stage 1	5 (9)
Stage 2	3 (5)
Stage 3	34 (60)
Stage 4	15 (26)
Histology, *n* (%)	
Serous	48 (84)
Endometroid	4 (7)
Mucinous	3 (5)
Carcinosarcoma	1 (2)
Other	1 (2)
Grade, *n* (%)	
Grade 1 and 2	15 (29)
Grade 3	36 (71)
Prior HRT, *n* (%)	20 (35)
Residual tumor, *n* (%)	
<1 cm	35 (62)
>1 cm	22 (38)
Response to platinum therapy[a], *n* (%)	
Sensitive	28 (49)
Resistant	13 (21)
Partial sensitive	12 (20)
Neoadjuvant therapy, *n* (%)	19 (35)
Recurrence	37 (65)
Death	21 (37)

FIGO International Federation of Gynecology, *HRT* hormone replacement therapy

[a] Sensitive: recurrence >12 months after completion of platinum-based first-line therapy. Resistant: recurrence ≤6 months after completion of platinum-based first-line therapy. Partially sensitive: recurrence 6–12 months after completion of platinum-based first-line therapy

Immunohistochemistry

Immunohistochemistry (IHC) was used to evaluate the PARP-1 protein expression. The archival representative ovarian cancer samples were derived from the Department of Pathology at Tampere University Hospital. The tumor sample blocks were cut into 3–4 μm thick sections using a standard microtome. For IHC staining, the slides were then deparaffinized, rehydrated in graded ethanol, and subsequently pretreated with a PT-Module (Lab Vision, Fremont, CA) at 98 °C for 15 min in 0.05 M TrisHCl buffer, pH 9.0 containing 0.001 M EDTA. The primary PARP-1 antibody (anti-PARP-1, Cat# sc-8007; Santa Cruz Biotechnology, Dallas, TX) at a dilution of 1:5000 was visualized with a PowerVision + polymer kit (Leica Biosystems Newcastle Ltd., Newcastle, UK) and diaminobenzidine as chromogen (DABImmPact, Vectorlabs, Burlingame, CA). The tissue sections were counterstained with hematoxylin (Mayer's hematoxylin, Oy FF-Chemicals Ab, Haukipudas, Finland), dehydrated, cleared, and mounted. Staining protocols were carried out with Autostainer 480 (Lab Vision, CA, USA) automated immunostainer. Placenta was used as a positive tissue control for PARP-1 staining. Positive and negative (primary antibody omitted) controls were included in each staining batch.

Immunostained sections were analyzed with Olympus System Microscope BX43. The assessment of PARP-1 staining was performed blinded in regard to the clinico-pathological information of the patients and PAR concentration by pharmacodynamic assay. In all tissue samples, PARP-1 nuclear staining intensity was scored semiquantitatively as low (negative or weak nuclear staining), intermediate (more pronounced or focally more intensive nuclear staining compared to low staining pattern), or strong (intensive uniform nuclear staining). The slides were independently scored by two different researchers, who were blinded to the patient medical history. For the statistical analysis, low and intermediate nuclear staining was combined as low PARP-1 staining and strong nuclear staining as high PARP-1 staining.

Statistical analyses

The clinico-pathological associations with variation in levels of PAR concentration and PARP-1 staining pattern were statistically evaluated. The comparison of the groups was made using the Student's *t* test, Mann-Whitney *U* test, and Fisher's exact test where appropriate. The Kaplan-Meier and Cox regression analyses were used to estimate the survival rates from the date of surgery (primary debulked patients) or from the date of the first dose of neoadjuvant therapy until the date of the event of interest. For progression-free and overall survival (PFS, OS), the event of interest was a recurrence or death, whichever occurred first. Patients alive at the last follow-up without a recurrence were censored at the last follow-up date. Version 22 of IBM SPSS statistics software (IBM Inc., Armonk, NY, USA) was used in statistical analyses. A p value less than 0.05 was considered significant.

Results

PAR concentration by ELISA pharmacodynamics assay

PAR concentration was available (i.e., PAR levels were measurable) from 51 out of 57 study patients (89 %). In fresh OC tumor tissue, PAR concentration levels ranged between 0.67 and 1024.68 pg/ml, with median and mean values of 203 and 311.1 (SD 292.1) pg/ml, respectively (Table 2). The cut-off level for high PARP activity was set to 203 pg/ml corresponding to median value.

Platinum sensitivity associated with high PAR concentration in the entire study cohort ($p = 0.022$; Fig. 1) and especially in the high-grade (i.e., grade 3) subgroup ($p = 0.017$). In turn, NACT associated with low levels of PAR ($p = 0.014$). The associations of levels of PAR concentration with clinico-pathological characteristics are summarized in Table 2.

In Kaplan-Meier analyses, high PAR concentration was associated with longer PFS than low concentration (32 vs 14 months, respectively, log-rank $p = 0.009$; Fig. 2). In Cox regression, low PAR concentration was associated with an increased risk of recurrence both in univariate (HR 2.4; 95 % CI 1.204–4.797) and

Table 2 Level of PAR concentration by ELISA pharmacodynamics assay in correlation with clinico-pathological characteristics

Characteristic	No. of patients	Median level of PAR concentration in pg/ml (IQR), $n = 51$	p value[a]
	51	203 (398.1)	
PARP staining by IHC			
Weak to moderate	24	197.7 (416.7)	0.82
Strong	26	196.3 (402.3)	
Age at surgery			
<Mean	26	289.2 (465.9)	0.13
>Mean	25	192.5 (348.7)	
BMI			
≤25	17	124.6 (426.3)	0.13
>25	33	243.5 (426.3)	
Grade			
1 and 2	15	279.1 (583.6)	0.12
3	36	163.4 (418.2)	
Prior use of HRT			
Yes	18	185.1 (280.6)	0.43
No	33	257.8 (430.8)	
CA125 after 3 cycles of CT or C alone			
Normal (≤35)	21	257.6 (478.2)	0.08
Abnormal (>35)	30	124.6 (391.0)	
Platinum sensitivity[b]			
Yes	26	312.6 (575.8)	0.022
No	22	117.6 (262.4)	
Platinum sensitivity[b] in high-grade subgroup			
Yes	16	404.7 (519.8)	0.017
No	19	81.4 (261.4)	
NACT			
Yes	16	87.1 (220.6)	0.01
No	35	292.5 (384.4)	
Recurrence[c]			
Yes	34	137.2 (388.7)	0.11
No	17	356.8 (450.6)	
Death[c]			
Yes	19	243.5 (379.9)	0.59
No	32	176.1 (459.8)	

IQR interquartile range, *IHC* immunohistochemistry, *HRT* hormone replacement therapy, *CT* carboplatin and taxane, *C* carboplatin, *NACT* neoadjuvant therapy

[a] Nonparametric Mann-Whitney *U* test

[b] Sensitivity defined as relapse or event-free follow-up time >12 months after completion of platinum-based first-line therapy

[c] Median duration of follow-up time in the study cohort was 31 months (range 2–50)

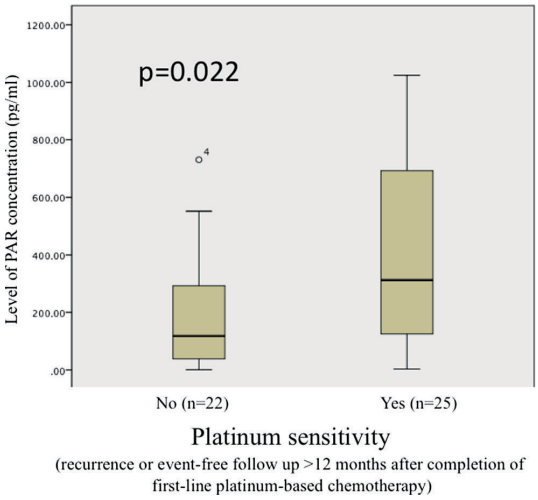

Fig. 1 Box plot diagram of PAR concentration levels according to platinum sensitivity ($p = 0.022$), Mann-Whitney *U* test; *horizontal lines* represent median levels of PAR concentration. Outlier is marked with a *circle*

multivariate analyses (Table 3), but not with increased risk of death (HR 1.12; 95 % CI 0.455–2.764).

PARP-1 immunohistochemistry

Representative tumor blocks were available from 56 patients. Half (or 28) of the tumors showed low/moderate PARP nuclear staining, while another half showed strong staining (Fig. 3). IHC PARP-1 staining pattern was not associated with PAR concentration by pharmacodynamic assay (Tables 2 and 4). A low PARP-1 immunohistochemistry staining associated significantly with normalized serum CA12-5 levels after three cycles of platinum-based chemotherapy ($p = 0.027$). The associations of PARP-1 IHC with clinico-pathological characteristics are summarized in Table 4.

In Kaplan-Meier analyses, a trend for association between low PARP-1 by IHC and longer PFS was detected (32 vs 15 months, log-rank $p = 0.061$; Fig. 4). However, in multivariate Cox regression analysis, low PARP-1 IHC was not associated with decreased risk of recurrence (Table 3). There was also no association with low PARP-1 IHC staining and overall survival (HR 0.724; 95 % CI 0.307–1.708).

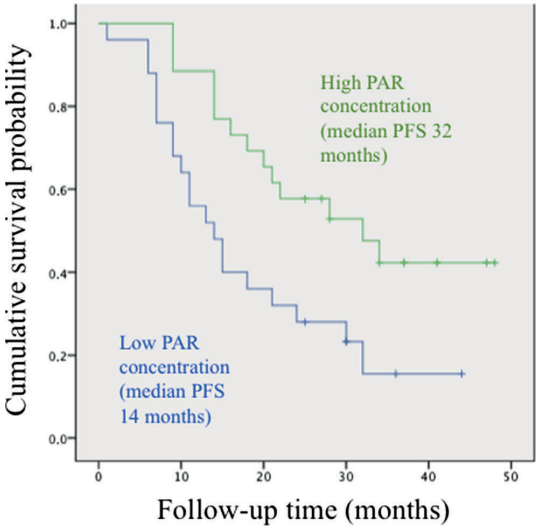

Fig. 2 Kaplan-Meier analysis of progression-free survival (PFS) according to median level of PAR concentration (log-rank $p = 0.009$). The cut-off level for high PAR concentration was set to 203 pg/ml (median value of PAR concentration). *Vertical lines* represent censored patients

Table 3 Risk factors for ovarian cancer progression in multivariate Cox regression analysis

Characteristic	HR	95 % CI	*p* value
Low PAR concentration[a]	2.521	1.132–5.615	0.024
Age at surgery (<mean)	0.993	0.946–1.042	0.769
Low PARP staining[b]	0.582	0.277–1.224	0.154
Primary surgery vs NACT	0.399	0.156–1.019	0.055
Low grade[c]	0.470	0.180–1.222	0.121
Local disease[d]	0.482	0.059–3.932	0.495
Non-optimal surgery	3.740	1.472–9.501	0.006

NACT neoadjuvant therapy, *HR* hazard ratio

[a] Cut-off point for low versus high PAR concentration, 203 pg/ml (median value of PAR concentration)

[b] Immunohistochemistry staining

[c] Grade 1 and 2 vs 3

[d] Stage I–II vs III–IV

Discussion

We report here an evaluation of PAR concentration as a surrogate marker for PARP enzyme activity in a prospective EOC patient cohort and PARP-1 IHC staining results of parallel archival tumor samples. Although there are previous data regarding *PARP1* gene expression [25] and PARP protein content by microarray technology and IHC in EOC [25, 27], to our knowledge, this is the first report of PARP enzyme activity measurement using fresh tumor samples from EOC patients in comparison with PARP-1 immunostaining pattern in corresponding archival tumor samples. The methodology used for measuring PARP activity (or PAR concentration) from cellular extracts is based on chemiluminescence and has previously been validated [30] and reported in a cohort of clinical breast cancer specimens [31]. PAR is the product of catalytic activity of PARP-1 enzyme and therefore measurement of PAR

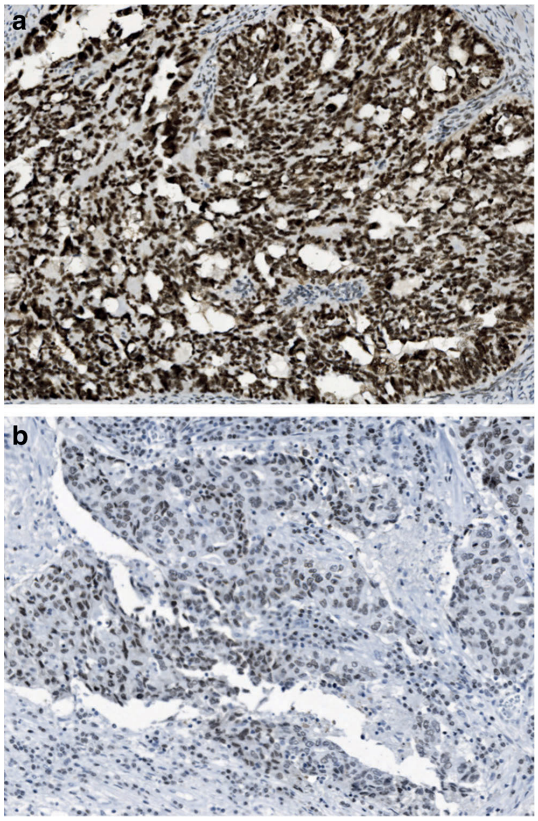

Fig. 3 Examples of PARP-1 immunohistochemistry staining in ovarian cancer tumor tissue. **a** Weak or moderate nuclear staining. **b** Strong nuclear staining

Table 4 PARP immunohistochemistry (IHC) staining and its correlation with clinico-pathological characteristics

Characteristic	PARP IHC weak to moderate staining, $n = 28$	PARP IHC strong staining, $n = 28$	p value[a]
PAR concentration[b], *n*			
Low[b]	12	12	1.00
High[b]	13	13	
Age at surgery, mean (SD)	65.96 (8.0)	66.46 (10.3)	0.841[c]
BMI, mean (SD)	26.24 (5.8)	27.15 (6.4)	0.583[c]
Grade, *n*			
1 and 2	9	7	0.768
3	19	21	
Prior use of HRT, *n*			
Yes	16	10	1.000
No	18	10	
CA12-5 after 3 cycles of CT or C alone, *n*			
Normal (≤35)	16	7	0.027
Abnormal (>35)	11	19	
Platinum sensitivity[d], *n*			
Yes	17	12	0.275
No	10	14	
Recurrence[e], *n*			
Yes	14	21	0.097
No	14	7	
NACT, *n*			
Yes	7	12	0.259
No	21	16	
Death[e], *n*			
Yes	10	11	1.000
No	18	17	

NACT neoadjuvant therapy, *HRT* hormone replacement therapy, *CT* carboplatin and taxane, *C* carboplatin

[a] Fisher's exact test for categorical characteristics (excluding age and BMI)

[b] Cut-off point for low versus high PAR concentration, 203 pg/ml (median value of PAR concentration)

[c] Student's *t* test for continuous variables showing normal distribution (age and BMI)

[d] Platinum sensitivity defined as relapse or event-free follow-up time >12 months after completion of platinum-based first-line therapy

[e] Median duration of follow-up time in the study cohort was 31 months (range 2–50)

product can be regarded as an indirect measurement of PARP-1 enzyme activity [25].

In the present study, high PARP activity in fresh frozen tumor tissue was associated with platinum sensitivity, which is generally accepted as a surrogate marker for HRd or *BRCA*ness [5]. Moreover, the association between high PARP activity and platinum sensitivity was even stronger in the high-grade subgroup. Both platinum sensitivity and HGSC in EOC have been considered as phenotypic characteristics of *BRCA*ness profile, further implying the possible association between HRd and high PARP activity [11]. Although in clinical setting high PARP activity has not been shown to predict response to PARP inhibitor therapy in OC [32], in mouse tumor models the current assay methodology has provided a valid biomarker for PARP inhibition [33]. Currently *BRCA* mutation status is considered as the primary biomarker for prediction of response to PARP inhibition, although it has been shown in a randomized clinical trial that also wild-type OC patients (i.e., *non-BRCA1/2* mutated) benefit from PARP inhibition with olaparib [20]. Functional tests that assess HR deficiency have been intensively studied in both ovarian cancer and triple-negative breast cancer [34, 35]. A HRd score based on patterns of genomic loss of heterozygosity has also been proposed to identify patients with HRd regardless of its etiology [36].

According to our results, high PARP activity was further translated into longer PFS, and low PARP activity was

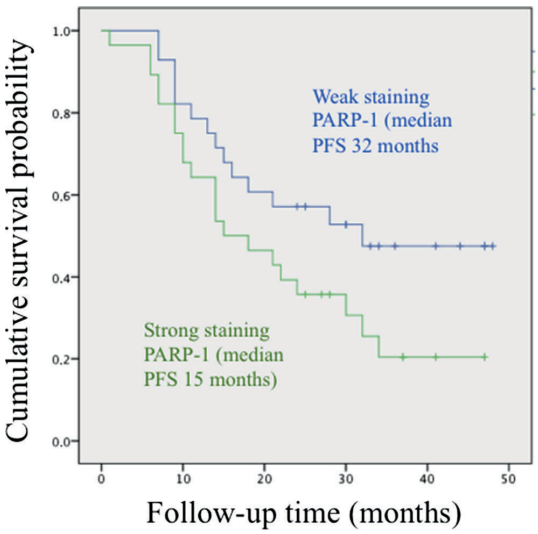

Fig. 4 Kaplan-Meier analysis of progression-free survival (PFS) according to PARP-1 immunohistochemistry staining (log-rank $p = 0.061$). *Vertical lines* represent censored patients

associated with early recurrence. In addition, the effect of PARP activity on PFS was found to be independent of tumor grade, stage, given NACT, or age of the patients. In our study, PARP activity and PARP-1 protein expression by IHC did not show any association. This discrepancy is somewhat difficult to explain, but it seems possible that a static assay like IHC is not optimal for evaluation of a dynamic repair mechanism of DNA single-strand breaks. Previous studies addressing PARP-1 expression in clinical OC material by IHC methods have reported contradictory findings and they also partly differ from the results presented here [27, 37]. High PARP-1 expression by IHC has been previously associated with complete response to initial chemotherapy but not with improved PFS [37]. On the other hand, weak PARP-1 staining by IHC has been associated with improved PFS and even OS [27]. In our data, there was also a trend for association between low PARP-1 by IHC and longer PFS and a significant association with a surrogate marker for platinum sensitivity, i.e., normalized CA12-5 level after three cycles of platinum-based chemotherapy. It is noteworthy that comparisons between these previous studies mentioned should, however, be made with caution since it is possible that the conflicting results are due to different methods used. For example, the previous IHC studies have used different primary PARP-1 antibodies with different scoring systems [27, 37]. It has also been suggested that the IHC assay used for PARP-1 determination detects not only functionally active PARP-1 but also inactive, automodified PARP-1 [31], and this could also explain inferiority of IHC methodology compared to enzymatic pharmacodynamic assay used in our study. Nevertheless, the optimal methodology to evaluate tumoral PARP-1 activity in clinical OC should be addressed in future studies [38, 39].

Another finding in the present study involves low PARP activity in samples obtained after NACT. Theoretically, NACT can lead to a low PARP activity in two ways. First, after platinum therapy, HRd tumors are dependent on BER and thus exploit PARP, decreasing its concentration. Second, NACT may induce active molecular changes in tumors, which are thereafter reflected as low PARP activity. The latter explanation is intriguing since several studies have suggested that NACT may induce platinum resistance [40]. However, it is somewhat challenging to interpret the finding of lower PARP activity after NACT, as we did not obtain sequential samples from patients before and after NACT, but all samples available are post-NACT. It is possible that the patients not eligible for upfront surgery and consequently, selected for NACT are a priori patients with worse prognosis. It is, however, worth mentioning that in a recent EOC study intratumoral PARP-1 expression by IHC was reduced after chemotherapy [26]. In that study, not only unmatched chemo-naïve and chemo-treated tumor samples were used but also nine PARP-1 positive cases with matched tumor samples before and after chemotherapy were included [26]. In our study, there was no association between NACT and PARP-1 IHC staining, but this may of course be due to a smaller sample size in our study or to differences in the antibodies used. In our study, we used an antibody recognizing highly conserved PARP-1 C-terminus containing the catalytic domain [41] instead of N-terminal domain [26]. As discussed before, the weakness of PARP-1 IHC seems to associate with its dependency on researcher-related methodological factors such as the antibody used, staining protocol and data analysis. Altogether, this preliminary finding warrants validation in a large cohort of sequential tumor samples obtained before and after NACT. If NACT truly affects PARP-1 protein expression or its enzymatic activity, it provides a relevant target for future research considering that PARP inhibitors are now used in the maintenance therapy of recurrent and heavily chemo-treated OC patients.

There are limitations to our study. First, the study cohort included a relatively small number of patients. This is due to the laborious and expensive collection of a prospective snap-frozen fresh tumor material as opposed to collection of, e.g., retrospective archival material. Despite this, we feel that our study cohort was comprehensive enough to be able to detect an association of PARP activity with response to chemotherapy, the outcome of our primary interest. Second, the fresh tissue samples for PARP analyses were not selected based on microscopic investigation but rather on a gross examination, which may have led to inclusion of non-tumor tissue in the analyses. Third, the genetic characterization (e.g., *BRCA* status) of the study cohort was not included. Our main scope, however, was to analyze PARP activity in fresh tumor tissue of EOC patients regardless of their *BRCA1/2* status.

In conclusion, we present the data of PARP pharmacodynamic assay and PARP IHC staining in EOC tumor tissue. We found an association between high PARP activity and platinum sensitivity and longer PFS, which is a novel finding. In addition, NACT seemed to be associated with low PARP activity. The IHC staining and pharmacodynamic assay findings did not associate, and PARP pharmacodynamic assay rather than PARP IHC seems to reflect better biologically significant PARP. Therefore, PARP pharmacodynamic assay may better identify patients with likely HRd tumors possibly benefiting from PARP inhibitors compared to PARP IHC.

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Compliance with ethical standards The study was carried out at the University of Tampere and Tampere University Hospital (TAUH), Tampere, Finland. The study protocol was approved by the Ethics Committee of TAUH (Identification code ETL-R11137, September 13, 2011).

Conflicts of interest None

References

1. George SHL, Shaw P. BRCA and early events in the development of serous ovarian cancer. Front Oncol. 2014;4:5.
2. Sonoda Y. Management of early ovarian cancer. Oncology. 2004;18:343–56. discussion 358, 361-2.
3. Piccart MJ, Lamb H, Vermorken JB. Current and future potential roles of the platinum drugs in the treatment of ovarian cancer. Ann Oncol. 2001;12:1195–203.
4. Martin LP, Hamilton TC, Schilder RJ. Platinum resistance: the role of DNA repair pathways. Clin Cancer Res. 2008;14:1291–5.
5. Wiedemeyer WR, Beach JA, Karlan BY. Reversing platinum resistance in high-grade serous ovarian carcinoma: targeting BRCA and the homologous recombination system. Front Oncol. 2014;4:34.
6. Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. Nature. 2011;474:609–15.
7. Cerbinskaite A, Mukhopadhyay A, Plummer ER, Curtin NJ, Edmondson RJ. Defective homologous recombination in human cancers. Cancer Treat Rev. 2012;38:89–100.
8. Tan DS, Rothermundt C, Thomas K, et al. "*BRCA*ness" syndrome in ovarian cancer: a case-control study describing the clinical features and outcome of patients with epithelial ovarian cancer associated with BRCA1 and BRCA2 mutations. J Clin Oncol. 2008;26:5530–6.
9. Bolton KL, Chenevix-Trench G, Goh C et al.; EMBRACE; kConFab Investigators; Cancer GenomeAtlas Research Network. Association between *BRCA1* and *BRCA2* mutations and survival in women with invasive epithelial ovarian cancer. JAMA. 2012;307:382–90.
10. Vencken PM, Kriege M, Hoogwerf D, et al. Chemosensitivity and outcome of BRCA1- and BRCA2-associated ovarian cancer patients after first-line chemotherapy compared with sporadic ovarian cancer patients. Ann Oncol. 2011;22:1346–52.
11. Konstantinopoulos PA, Spentzos D, Karlan BY, Taniguchi T, Fountzilas E, Francoeur N, et al. Gene expression profile of *BRCA*ness that correlates with responsiveness to chemotherapy and with outcome in patients with epithelial ovarian cancer. J Clin Oncol. 2010;28:3555–61.
12. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in *BRCA* mutant cells as a therapeutic strategy. Nature. 2005;434(7035):917–21.
13. Parsons JL, Dianova II, Allinson SL, Dianov GL. Poly(ADP-ribose) polymerase-1 protects excessive DNA strand breaks from deterioration during repair in human cell extracts. FEBS J. 2005;272(8):2012–2.
14. Veuger SJ, Curtin NJ, Smith GC, Durkacz BW. Effects of novel inhibitors of poly(ADP-ribose) polymerase-1 and the DNA-dependent protein kinase on enzyme activities and DNA repair. Oncogene. 2004;23:7322–9.
15. Metzger MJ, Stoddard BL, Monnat Jr RJ. PARP-mediated repair, homologous recombination, and back-up non-homologous end joining-like repair of single-strand nicks. DNA Repair. 2013;2:529.
16. Shen Y, Aoyagi-Scharber M, Wang B. Trapping PARP. J Pharmacol Exp Ther. 2015;353(3):446–57. doi:10.1124/jpet.114.222448.
17. Fong PC, Boss DS, Yap TA, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. N Engl J Med. 2009;361:123–34.
18. Audeh MW, Carmichael J, Penson RT, et al. Oral poly(ADP-ribose) polymerase inhibitorx olaparib in patients with *BRCA1* or *BRCA2* mutations and recurrent ovarian cancer: a proof-of-concept trial. Lancet. 2010;376:245–51.
19. Ledermann J, Harter P, Gourley C, et al. Olaparib maintenance therapy in platinum-sensitive relapsed ovarian cancer. N Engl J Med. 2012;366:1382–92.
20. Ledermann J, Harter P, Gourley C, et al. Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: a preplanned retrospective analysis of outcomes by *BRCA* status in a randomized phase 2 trial. Lancet Oncol. 2014;15:852–61.
21. McCabe N, Turner NC, Lord CJ, et al. Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP- ribose) polymerase inhibition. Cancer Res. 2006;66:8109–15.
22. Gottipati P, Vischioni B, Schultz N, et al. Poly(ADP-ribose) polymerase is hyperactivated in homologous recombination-defective cells. Cancer Res. 2010;70(13):5389–98.
23. Liu JF, Konstantinopoulos PA, Matulonis UA. PARP inhibitors in ovarian cancer: current status and future promise. Gynecol Oncol. 2014;133(2):362–9.
24. Pennington KP, Walsh T, Harrell MI, et al. Germline and somatic mutations in homologous recombination genes predict platinum response and survival in ovarian, fallopian tube, and peritoneal carcinomas. Clin Cancer Res. 2014;20:764–75.
25. Ossovskaya V, Koo IC, Kaldjian EP, Alvares C, Sherman BM. Upregulation of Poly(ADP-Ribose) polymerase-1 (PARP1) in triple-negative breast cancer and other primary human tumor types. Genes Cancer. 2010;1:812–2.
26. Marques M, Beauchamp MC, Fleury H, Laskov I, Qiang S, Pelmus M, et al. Chemotherapy reduces PARP1 in cancers of the ovary: implications for future clinical trials involving PARP inhibitors. BMC Med. 2015;13:217.
27. Gan A, Green AR, Nolan CC, Martin S, Deen S. Poly(adenosine diphosphate-ribose) polymerase expression in BRCA-proficient ovarian high-grade serous carcinoma; association with patient survival. Hum Pathol. 2013;44:1638–47.

28. Denkert C, Sinn BV, Issa Y, Maria Müller B, Maisch A, Untch M, et al. Prediction of response to neoadjuvant chemotherapy: new biomarker approaches and concepts. Breast Care. 2011;6(4):265–72.
29. Garg K, Levine DA, Olvera N, Dao F, Bisogna M, Secord AA, et al. BRCA1 immunohistochemistry in a molecularly characterized cohort of ovarian carcinomas. Am J Surg Pathol. 2013;37:138–46.
30. Kinders RJ, Hollingshead M, Khin S, Rubinstein L, Tomaszewski JE, Doroshow JH, et al. Preclinical modeling of a phase 0 clinical trial: qualification of a pharmacodynamic assay of poly(ADP-ribose) polymerase in tumor biopsies of mouse xenografts. Clin Cancer Res. 2008;14:6877–85.
31. Jacot W, Thezenas S, Senal R, et al. *BRCA1* promoter hypermethylation, 53BP1 protein expression and PARP-1 activity as biomarkers of DNA repair deficit in breast cancer. BMC Cancer. 2013;13:523.
32. Burgess M, Puhalla S. *BRCA 1/2*-mutation related and sporadic breast and ovarian cancers: more alike than different. Front Oncol. 2014;4:19.
33. Liu X, Palma J, Kinders R, Shi Y, et al. An enzyme-linked immunosorbent poly(ADP-ribose) polymerase biomarker assay for clinical trials of PARP inhibitors. Anal Biochem. 2008;381:240–7.
34. Naipal KA, Verkaik NS, Ameziane N, et al. Functional ex vivo assay to select homologous recombination-deficient breast tumors for PARP inhibitor treatment. Clin Cancer Res. 2014;20:4816–26.
35. Shah MM, Dobbin ZC, Nowsheen S, Wielgos M, Katre AA, Alvarez RD, et al. An ex vivo assay of XRT-induced Rad51 foci formation predicts response to PARP-inhibition in ovarian cancer. Gynecol Oncol. 2014;134:331–7.
36. Abkevich V, Timms KM, Hennessy BT, Potter J, Carey MS, Meyer LA, et al. Patterns of genomic loss of heterozygosity predict homologous recombination repair defects in epithelial ovarian cancer. Br J Cancer. 2012;107(10):1776–82.
37. Godoy H, Mhawech-Fauceglia P, Beck A, Miller A, Lele S, Odunsi K. Expression of poly (adenosine diphosphate-ribose) polymerase and p53 in epithelial ovarian cancer and their role in prognosis and disease outcome. Int J Gynecol Pathol. 2011;30:139–44.
38. von Minckwitz G, Muller BM, Loibl S, Budczies J, Hanusch C, Darb-Esfahani S, et al. Cytoplasmic poly(adenosine diphosphate-ribose) polymerase expression is predictive and prognostic in patients with breast cancer treated with neoadjuvant chemotherapy. J Clin Oncol. 2011;29:2150–7.
39. Rojo F, Garcia-Parra J, Zazo S, Tusquets I, Ferrer-Lozano J, Menendez S, et al. Nuclear PARP-1 protein overexpression is associated with poor overall survival in early breast cancer. Ann Oncol. 2012;23:1156–64.
40. Sato S, Itamochi H. Neoadjuvant chemotherapy in advanced ovarian cancer: latest results and place in therapy. Ther Adv Med Oncol. 2014;6:293–304.
41. Muthurajan UM, Hepler MR, Hieb AR, Clark NJ, Kramer M, Yao T, et al. Automodification switches PARP-1 function from chromatin architectural protein to histone chaperone. Proc Natl Acad Sci U S A. 2014;111:12752–7.

PUBLICATION

III

Expression analysis of platinum sensitive and resistant epithelial ovarian cancer patient samples reveals new biomarkers for targeted therapies

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Expression Analysis of Platinum Sensitive and Resistant Epithelial Ovarian Cancer Patient Samples Reveals New Candidates for Targeted Therapies[1]

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Abstract

Ovarian cancer has the highest mortality rate of all gynecologic malignancies. Identification of new biomarkers is highly needed due to its late diagnosis and high recurrence rate. The objective of this study was to identify mechanisms of therapy resistance and potential biomarkers by analyzing mRNA and protein expression from samples derived from patients with platinum-sensitive and -resistant ovarian cancer (total cohort n = 53). The data revealed new candidates for targeted therapies, such as GREB1 and ROR2. We showed that the development of platinum resistance correlated with upregulation of ROR2, whereas GREB1 was downregulated. Moreover, we demonstrated that high levels of ROR2 in platinum-resistant samples were associated with upregulation of Wnt5a, STAT3 and NF-kB levels, suggesting that a crosstalk between the non-canonical Wnt5a-ROR2 and STAT3/NF-kB signaling pathways. Upregulation of ROR2, Wnt5a, STAT3 and NF-kB was further detected in a platinum-resistant cell-line model. The results of the present study provided insight into molecular mechanisms associated with platinum resistance that could be further investigated to improve treatment strategies in this clinically challenging gynecological cancer.

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Introduction

Epithelial ovarian cancer (EOC) accounts for the majority of mortality from gynecological cancers, with diagnosis often at a late stage. Currently, the golden standard of treatment is primary debulking surgery (PDS) followed by platinum-based chemotherapy [1]. Although most patients initially respond to chemotherapy, cancer cells will eventually develop resistance leading to relapse [2]. Despite intensive efforts to improve targeted therapy in EOC, the five-year survival rate is still only 30% for advanced disease [3]. Therefore, increased knowledge about mechanisms of platinum resistance in EOC treatment is needed in search for cure.

Genetically complex and unstable high-grade serous ovarian cancer subtype (HGSC), accounting for approximately 50–70% of EOC, represents the most aggressive histological subtype [4]. A large-scale integrated genomic data analysis for HGSC identified TP53 mutations in almost 96% of tumors. Recurrent somatic mutations were found in nine other genes including NF1, BRCA1, BRCA2,

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RB1 and CDK12, as well as DNA copy number aberrations and promoter methylation events, indicating biological and molecular heterogeneity that should be considered when developing novel therapeutic strategies [5].

While only 10%–15% of ovarian cancer patients carry BRCA1 or BRCA2 mutations in their germline, ~50% of ovarian cancers exhibit a defect in the homologous recombination (HR) repair of DNA [6]. PARP-1 enzyme became an attractive target for chemotherapeutics for its crucial function in single-strand breaks (SSBs) DNA repair mechanism through base excision repair (BER) pathway [7]. The concept of synthetic lethality has been used in genetic studies to determine functional interactions and compensation among genes for decades and has also been exploited in the development of PARP (Poly (ADP-ribose) polymerase) inhibitors [8]. The responsiveness to platinum and PARP inhibitors associates with so called *BRCAness* profile showing independent prognostic value [9,10].

The chemotherapy resistance can arise due to multiple mechanisms, such as drug target alteration, re-activation or amplification of the oncogenic pathway, activation of parallel pathways, increased DNA damage tolerance/repair, and deregulation of growth factor receptors among others [11]. Deregulation of apoptosis and altered phosphorylation (intracellular signaling), as well as metabolic pathways represent the two main biological processes responsible for oncogene-mediated drug resistance in ovarian cancer [12]. In this context, activation of PI3K/AKT cell survival pathway plays a pivotal role with NF-kB and STAT3 as the main mediations of these intracellular events. On the other hand, tumor suppressor genes such as BRCA1, BRCA2, MLH1 and p21 contribute to ovarian cancer drug resistance via alterations in the DNA damage and repair mechanisms, whereas RASSF1, TP53 and TP73 impair the apoptotic machinery for the same outcome [13]. Epithelial-to-mesenchymal transition (EMT) has also been implicated in HGSC invasiveness and chemoresistance, and in vitro studies using ovarian cancer cell lines have shown that more aggressive, mesenchymal-type cells are more resistant to cisplatin treatment [14]. An important signaling cascade involved in EMT is the Wnt signaling, with increasing evidence suggesting that β-catenin-independent pathway via Wnt5a/ROR1/ROR2 has a critical role in EMT and chemoresistance [15–18]. Consequently, therapies targeting these pathways may offer means to overcome drug resistance.

Active and also productive research in the field of cancer therapy has led to an improved understanding of the molecular mechanisms, providing insight into the development of cancer. This new data has led to the development of new treatment options for cancer patients, including targeted therapies and associated biomarker tests that can select which patients are most likely to respond [19]. The aim of this study was to identify candidate genes and their molecular pathways involved in the pathogenesis of ovarian cancer associating with platinum resistance. Overcoming the paucity of obtaining large collection of tumor samples available for molecular profiling, we investigated differences in mRNA and protein expression between ovarian cancer samples derived from two clinically and molecularly distinct patient cohorts namely high PARP/platinum-sensitive and low PARP/platinum-resistant HGSC cohorts. This comparison aimed at distinction of two cohorts with extremely different clinical behavior. Finally, this analysis led to identification of GREB1 and ROR2 that showed significant differential expression profile between the two groups. Our data suggest new predictive biomarkers for ovarian cancer drug resistance development warranting further investigations.

Materials and Methods

Study Cohort and Tissue Samples

The study was carried out at the University of Tampere and Tampere University Hospital (TAUH), Tampere, Finland. The study protocol was approved by the Ethics Committee of TAUH (identification code ETL-R11137).

The microarray study cohort consisted of 12 HGSC patients who participated in a prospective study addressing PARP enzyme activity in fresh ovarian cancer tumor samples [20]. The selection of this patient subcohort was based on PARP values (high PARP/low PARP, cut off 203 pg/ml, which corresponded to the median value of PARP) and platinum sensitivity/resistance (treatment response), with no differences in respect of age and FIGO (International Federation of Gynecology). Platinum sensitivity was defined as no recurrence within 12 months after the completion of first-line platinum-based chemotherapy. Patient characteristics are presented in Table 1.

The validation cohort of the microarray data consisted of all the patients (n = 53) that participated in the previous prospective study [20]. The median follow-up time of patients was 31 months. The validation cohort is described in Table 2.

For further investigation of ROR2 in EOC, a retrospective subcohort was chosen from the study cohorts described above consisting of a subgroup of patients who had not received NACT (neoadjuvant chemotherapy) and were divided in two categories based on treatment response, i.e. either platinum-sensitive or platinum-resistant (Table 3).

The tumor tissue samples were collected at surgery, two samples approximately 0.5 cm were chosen at the operation room from macroscopically visible tumor and were snap-frozen with liquid nitrogen and stored in −70 °C. The findings from the corresponding archival surgical tumor specimens were assessed by experienced pathologists as part of routine diagnostics at the Department of Pathology at TAUH.

Table 1. Characteristics of the Study Patients in the Microarray Cohort (n = 12)

Characteristic	Platinum Sensitive * n (%)	Platinum Resistant * n (%)
All	6	6
PDS †	5	2
NACT ‡	1	4
PARP § low	0	6
PARP § high	6	0
Age		
mean (SD)	65	62
median (range)	63 (46–78)	62 (55–79)
Grade 3 ¶	6 (100%)	6 (100%)
Stage #		
FIGO st I	0	0
FIGO st II	0	0
FIGO st III and IV	6	6
Histology		
serous	6 (100%)	6 (100%)
PFS ** (months)	28	3.5

* Sensitivity defined as relapse or event-free interval> 12 months after completion of platinum based 1st line therapy.

† PDS - primary debulking surgery.

‡ NACT - neoadjuvant therapy.

§ PARP - PARP activity in fresh frozen tumor tissue was assessed by an enzymatic chemiluminescense assay in a previous study [20]; the cut-off level for high PARP activity was set to 203 pg/ml corresponding to median value.

¶ Grade – Grade 3 represents high grade tumors.

FIGO - International Federation of Gynecology.

** PFS - progression free survival.

Table 2. Characteristics of the Study Patients in the Validation Cohort (n = 53)

Characteristic	
Patients in study, n	53
Age at surgery, yrs. (SD)	66 (9.3)
BMI, mean (SD)	26.8 (6.2)
Median follow-up, months (range)	31 (2–50)
Ca 125 level (kU/L) before treatment, median (range)	523 (30–4728)
FIGO* Stage, n (%)	
Stage 1	2 (3,7%)
Stage 2	3 (5,6%)
Stage 3	34 (64,3%)
Stage 4	14 (26,4%)
Histology, n (%)	
Serous	46 (86,8)
Endometroid	4 (7,6%)
Papillar	2 (3,7%)
Mucinous	0 (0%)
Carcinosarcoma	0 (0%)
Transitional cell	1 (1,9%)
Grade†, n (%)	
Grade 1 and 2	10 (18%)
Grade 3	43 (82%)
Sensitivity to platinum therapy‡, n (%)	
Sensitive	25 (47,2%)
Resistant	15 (28,3%)
Partial sensitive	13 (24,5%)
Neoadjuvant therapy, n (%)	19 (36%)
Recurrence	36 (68%)
Death	22 (42%)

* FIGO = International Federation of Gynecology.

† Grade – Grade 3 represents high grade tumors, Grade 1 and 2 low grade tumors.

‡ Sensitive: Recurrence >12 months after completion of platinum-based 1st line therapy; Resistant: Recurrence ≤6 months after completion of platinum-based 1st line therapy; Partially sensitive: Recurrence 6–12 months after completion of platinum-based 1st line therapy.

mRNA Microarray

Total RNA from ovarian cancer fresh frozen samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The quality and integrity of the RNA was assessed using Fragment Analyzer parallel capillary electrophoresis (Advanced Analytical Technologies, Ankeny, IA, USA). The RNA was subsequently labeled and hybridized using the Agilent gene expression microarray kit (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instruction. Briefly, the RNA from the clinical samples was labeled with Cy3 fluorochrome and subsequently co-hybridized with Cy5 labeled Xpress Ref TM Human Universal Reference Total RNA (SuperArray Bio-science Corporation) as a control. The hybridization was carried out for 21 hours on Agilent 4X44K human gene expression array slides. The slides were subsequently scanned on an Agilent C scanner and raw data were extracted using the Agilent Feature Extraction software ver. 11.0.1.1 and quantile normalized. A fold-change cutoff of 2 was used to determine differential mRNA expression as well as q-value and signal intensity.

Table 3. Characteristics of the study patients in the ROR investigation subcohort (n = 30)

Characteristic	
Patients in study, n	30
Age at surgery, yrs mean (SD)	66 (9.3)
Median follow-up, months (range)	31 (2–50)
FIGO* Stage, n (%)	
Stage 1	2 (6,7%)
Stage 2	4 (14%)
Stage 3	17 (56%)
Stage 4	7(23,3%)
Histology, n (%)	
Serous	22(73,3%)
Endometroid	2 (6,7%)
Papillar	2 (6,7%)
Mucinous	2 (6,7%)
Carcinosarcoma	1 (3,3%)
Transitional cell	1 (3,3%)
Grade†, n (%)	
Grade 1 and 2	8 (26,7%)
Grade 3	22 (73,3%)
Response to platinum therapy‡, n (%)	
Sensitive	20 (66,7%)
Resistant	10 (33,3%)
Recurrence	20 (66,7%)
Death	9 (30%)

* FIGO = International Federation of Gynecology.

† Grade – Grade 3 represents high grade tumors, Grade 1 and 2 low grade tumors.

‡ Sensitivity defined as relapse or event-free follow up time> 12 months after completion of platinum based 1st line therapy.

qRT-PCR

Putatively differentially expressed genes (*ROR2, CAST, ATP6V1D, GUCY1A3, TMOD1, MYCN, DLK1, PLEKHG4B, GREB1, B4GALNT4, SLC35F3, PTCH2, TNNC1, BNC1*) were selected from the array results based on fold change, q-value, signal intensity and literature data and were validated by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA from ovarian cancer fresh frozen tumor samples were extracted with TRIzol as described above and were reverse transcribed using random hexamere primers and MultiScribe reverse transcriptase (Thermo Fischer Scientific, Waltham, MA, USA). Quantitative Real Time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fischer Scientific) on a BioRad CFX96 ™ Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA, USA). Each sample was run in duplicate and expression values were normalized against the TATA-binding protein (TBP). The primer sequences for the two validated genes are as follows:

GREB1 fw	5'ATGGGAAATTCTTACGCTGGAC
GREB1 rev	5'CACTCGGCTACCACCTTCT
ROR2 fw	5'GTGCGGTGGCTAAAGAATGAT
ROR2 rev	5'ATTCGCAGTCGTGAACCATATT
TBP fw	5'GAATATAATCCCAAGCGGTTTG
TBP rev	5'ACTTCACATCACAGCTCCCC

Western blotting

Frozen tumor pieces were thawed, washed 2X with cold PBS and pestered to lyse in lysis buffer (50 mM Tris–HCl pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton-x-100, 50 mM NaF) supplemented with protease and phosphatase inhibitor cocktails (Bimake, Houston, TX, USA). Lysates were mixed with 4X Laemmli loading buffer and subjected to SDS-PAGE gel electrophoresis and Western blotting. The primary antibodies used were as following: pAkt S473 (#4060), pMEK1/2 S217/221 (#9121), NF-κB p65 (#6956), pPI3K p85 Y458/p55 Y199 (#4228), PI3K p85α (#13666), Rac-1 (#4651), pSTAT3 Y705 (#9145), STAT3 (#9139), Wnt5a/b (#2530) (Cell Signaling Technology, Danvers, MA, USA); Akt (#sc-5298), Bcl-2 (#sc-7382), MEK1/2 (#sc-6250), β-tubulin (#sc-166,729) (Santa Cruz, Dallas, TX, USA); ROR1 6D4 (Dr. Riddel lab, ref. Balakrishana et al. 2016); ROR2 (#565550, BD Biosciences, San Jose, CA, USA). Secondary antibodies: IRDye® 800CW Donkey anti-Mouse IgG, or IRDye® 680RD Donkey anti-Rabbit IgG (LI-COR, Lincoln, NE, USA). Blots were scanned and quantified using Odyssey CLx and Image Studio

software (LI-COR). Protein quantification was normalized to β-tubulin expression level for each sample.

Cell Culture

A2780 and A2780cis cells were purchased from Merck & Company Inc. (Kenilworth, NJ, USA) and cultured according to manufacturer's recommendations. The A2780 line was maintained in medium containing 1 μM cisplatin (Selleckchem, Munich, Germany). The cisplatin EC50 response of A2780 and A2780cis cells was validated by incubating cells with increasing concentration of cisplatin for 3 days and cell viability was determined using CellTiterGlo (CTG) Assay (Promega, USA) according to manufacturer's instructions.

qRT-PCR of the Ovarian Cancer Cell Lines

RNA was collected from A2780 and A2780cis using TRI Reagent® (Molecular Research Center Inc. Cincinnati, OH, USA) according to the manufacturer's protocol. qRT-PCR was performed as described for the ovarian cancer clinical samples. Each cell line was run in 4 replicates and the expression of ROR2 and GREB1 was normalized against TBP.

Statistical Analysis

The statistical analysis of mRNA microarray data was implemented in R using packages limma and preprocessCore of Bioconductor project [21–23]. Data was quantile normalized and probe sets were summarized by choosing the probes with the highest average expression [22]. Using limma approach, differential expression was identified between patients who had low PARP value and were platinum resistant, and patients who had high PARP value and were platinum sensitive [23]. P-values were obtained by the empirical Bayes moderated t-test. A fold-change cutoff of 2 was used to determine differential mRNA expression. The results were

Table 4. The 50 Most Upregulated mRNAs in High PARP and Platinum Sensitive OC Patient Samples in Comparison to low PARP and Platinum Resistant Samples.

Gene name	Gene description	log2-fold change	p-value	q-value
COLEC11	collectin subfamily member 11	3.68E+ 00	1.29E-02	0.2588936
MYCN	**MYCN proto-oncogene. bHLH transcription factor**	**3.61E+ 00**	**2.74E-04**	**0.1050305**
ESM1	endothelial cell specific molecule 1	3.18E+ 00	1.64E-04	0.1028588
IGF1R	insulin like growth factor 1 receptor	3.08E+ 00	5.29E-03	0.211962
TNNC1	**troponin C1. slow skeletal and cardiac type**	**3.04E+ 00**	**1.25E-05**	**0.0472319**
LGSN	lengsin. Lens protein with glutamine synthetase domain	3.02E+ 00	1.25E-02	0.2588936
LOC100134423	uncharacterized LOC100134423	2.97E+ 00	2.71E-04	0.1050305
DLK1	**delta like non-canonical Notch ligand 1**	**2.89E+ 00**	**4.17E-03**	**0.2013983**
CRYGC	crystallin gamma C	2.86E+ 00	2.37E-03	0.1822358
A_33_P3286709	NA	2.82E+ 00	9.15E-05	0.0864965
PLEKHG4B	**pleckstrin homology and RhoGEF domain containing G4B**	**2.82E+ 00**	**1.17E-03**	**0.1525447**
TSPAN8	tetraspanin 8	2.78E+ 00	2.99E-02	0.3064189
ENPP6	ectonucleotide pyrophosphatase/phosphodiesterase 6	2.73E+ 00	1.20E-02	0.2563874
FLJ30901	uncharacterized protein FLJ30901	2.60E+ 00	3.26E-04	0.1050305
PROM1	prominin 1	2.59E+ 00	3.02E-02	0.3079072
COL22A1	collagen type XXII alpha 1 chain	2.58E+ 00	2.64E-03	0.1844765
KIAA1324	KIAA1324	2.58E+ 00	5.48E-04	0.1261184
PTCH2	**patched 2**	**2.57E+ 00**	**1.93E-05**	**0.0472319**
SLC35F3	**solute carrier family 35 member F3**	**2.54E+ 00**	**3.87E-05**	**0.0596903**
GABRG3	gamma-aminobutyric acid type A receptor gamma3 subunit	2.51E+ 00	4.50E-05	0.0628517
LOC613266	uncharacterized LOC613266	2.50E+ 00	4.89E-02	0.3485897
LCE1E	late cornified envelope 1E	2.49E+ 00	2.28E-04	0.1050305
B4GALNT4	**beta-1.4-N-acetyl-galactosaminyltransferase 4**	**2.49E+ 00**	**1.24E-05**	**0.0472319**
STC2	stanniocalcin 2	2.48E+ 00	2.86E-04	0.1050305
FOXL2NB	FOXL2 neighbor	2.48E+ 00	1.04E-01	0.4447586
AK124496	NA	2.47E+ 00	2.78E-02	0.3020977
CU677518	NA	2.47E+ 00	2.82E-06	0.0275612
NM_130777	NA	2.45E+ 00	1.10E-02	0.2525629
NR_102701	NA	2.43E+ 00	2.11E-03	0.1762016
NSG1	neuronal vesicle trafficking associated 1	2.43E+ 00	1.05E-03	0.1525447
STAR	steroidogenic acute regulatory protein	2.41E+ 00	5.08E-03	0.2110634
MCTS2P	malignant T-cell amplified sequence 2. pseudogene	2.40E+ 00	5.43E-03	0.2134259
TRABD2A	TraB domain containing 2A	2.36E+ 00	4.89E-03	0.2078187
DEPTOR	DEP domain containing MTOR interacting protein	2.35E+ 00	4.70E-04	0.125416
CLEC4GP1	C-type lectin domain family 4 member G pseudogene 1	2.35E+ 00	4.56E-03	0.2021535
LINC01405	long intergenic non-protein coding RNA 1405	2.33E+ 00	6.95E-04	0.1391319
COL2A1	collagen type II alpha 1 chain	2.32E+ 00	1.82E-02	0.2816697
HS6ST2	heparan sulfate 6-O-sulfotransferase 2	2.31E+ 00	2.15E-02	0.2871745
PRAME	preferentially expressed antigen in melanoma	2.30E+ 00	4.32E-05	0.0628517
LINC02398	long intergenic non-protein coding RNA 2398	2.29E+ 00	1.08E-05	0.0472319
GJB7	gap junction protein beta 7	2.29E+ 00	3.18E-02	0.3122712
PLCXD3	phosphatidylinositol specific phospholipase C X domain containing 3	2.28E+ 00	6.03E-02	0.3707384
ERVI-1	endogenous retrovirus group I member 1	2.27E+ 00	1.28E-02	0.2588936
ZNF556	zinc finger protein 556	2.27E+ 00	1.46E-06	0.0213888
NTS	neurotensin	2.26E+ 00	6.56E-02	0.3828556
BU963192	NA	2.25E+ 00	7.17E-05	0.0751488
GMNC	geminin coiled-coil domain containing	2.24E+ 00	1.52E-02	0.2689731
A_33_P3274001	NA	2.24E+ 00	9.36E-05	0.0864965
CRYGD	crystallin gamma D	2.24E+ 00	2.58E-02	0.2964626
GREB1	**growth regulation by estrogen in breast cancer 1**	**2.22E+ 00**	**1.22E-03**	**0.1542519**

mRNAs selected for further validation are shown in bold.

interpreted by principal component analysis [24] and hierarchical clustering.

qRT-PCR data were analyzed using the Student's t-test and Mann–Whitney U test where appropriate. The Kaplan–Meier regression analyses were used to estimate the survival rates from the date of surgery (primary debulked patients) or from the date of the first dose of neoadjuvant therapy until the date of the event of interest. For progression-free survival (PFS), the event of interest was a recurrence or death, whichever occurred first. Patients alive at the last follow-up without a recurrence were censored at the last follow-up date. Statistical analysis was performed using GraphPad Prism 6 software for Windows (GraphPad Software Inc., La Jolla, CA, USA). A p-value less than 0.05 was considered significant.

Oncomine (https://www.oncomine.org/resource/login.html) and Kaplan–Meier plotter (http://kmplot.com/analysis/index.php?p=service&cancer=ovar) databases were searched for gene expression and survival data.

Results

Microarray Analysis of HGSC Patient Samples

In order to identify differentially expressed genes between ovarian cancer samples with platinum-sensitivity with high PARP levels and samples with platinum resistance with low PARP levels (n = 12), gene expression microarray was performed on total RNA isolated from the freshly frozen tumor samples. The analysis of gene expression showed a total of 3001 differentially expressed genes between the two comparison groups when a log fold change cutoff 2 was implemented. In this comparison, 1463 genes were downregulated and 1538 genes were upregulated. 50 most upregulated and 50

Table 5. The 50 Most Downregulated mRNAs in High PARP and Platinum Sensitive OC Patient Samples in Comparison to Low PARP and Platinum Resistant Samples

Gene Name	Gene Description	Log2-Fold Change	p-Value	q-Value
EFEMP1	EGF containing fibulin extracellular matrix protein 1	−3.85E+ 00	1.50E-03	0.1587368
FAP	fibroblast activation protein alpha	−3.66E+ 00	9.07E-04	0.1525447
TMOD1	**tropomodulin 1**	**−3.41E+ 00**	**2.92E-08**	**0.0008577**
BNC1	**basonuclin 1**	**−3.36E+ 00**	**2.74E-04**	**0.1050305**
ENST00000453673	NA	−3.22E+ 00	2.22E-02	0.2877726
ENST00000411475	NA	−3.18E+ 00	2.30E-02	0.2898232
SCRG1	stimulator of chondrogenesis 1	−3.18E+ 00	1.64E-03	0.1627023
PTGFR	prostaglandin F receptor	−3.04E+ 00	1.05E-03	0.1525447
POSTN	periostin	−3.01E+ 00	1.10E-02	0.2525629
ENST00000390237	NA	−2.97E+ 00	1.54E-02	0.2695379
BF175071	NA	−2.92E+ 00	5.12E-02	0.3538495
GUCY1A3	**guanylate cyclase 1 soluble subunit alpha**	**−2.89E+ 00**	**5.68E-05**	**0.0723946**
ENST00000390628	NA	−2.87E+ 00	4.44E-03	0.2021325
CXCL13	C-X-C motif chemokine ligand 13	−2.87E+ 00	1.02E-02	0.2487313
ACKR4	atypical chemokine receptor 4	−2.86E+ 00	9.73E-05	0.0864965
IGLL5	immunoglobulin lambda like polypeptide 5	−2.86E+ 00	3.46E-02	0.318167
AREG	amphiregulin	−2.83E+ 00	1.43E-02	0.2670241
A_33_P3251412	NA	−2.81E+ 00	1.46E-02	0.2682963
FYB1	FYN binding protein 1	−2.79E+ 00	2.51E-03	0.1843078
CD38	CD38 molecule	−2.78E+ 00	1.54E-03	0.1587368
ENST00000468879	NA	−2.77E+ 00	4.36E-02	0.3380632
ENST00000390252	NA	−2.73E+ 00	1.49E-02	0.2682963
JCHAIN	joining chain of multimeric IgA and IgM	−2.70E+ 00	6.61E-03	0.2195342
ENST00000390547	NA	−2.69E+ 00	5.85E-03	0.2163421
MEDAG	mesenteric estrogen dependent adipogenesis	−2.69E+ 00	4.49E-02	0.3403785
CNR1	cannabinoid receptor 1	−2.68E+ 00	9.85E-03	0.2471112
PRRX1	paired related homeobox 1	−2.68E+ 00	7.45E-04	0.1427284
NR4A3	nuclear receptor subfamily 4 group A member 3	−2.67E+ 00	2.00E-02	0.2850432
CH25H	cholesterol 25-hydroxylase	−2.65E+ 00	2.21E-03	0.1762175
AB363267	NA	−2.65E+ 00	4.23E-02	0.3347815
ENST00000426099	NA	−2.63E+ 00	4.11E-02	0.331398
CCL18	C-C motif chemokine ligand 18	−2.57E+ 00	1.16E-04	0.0898927
THBS2	thrombospondin 2	−2.57E+ 00	3.91E-02	0.327707
ENST00000410078	NA	−2.56E+ 00	5.30E-02	0.3580372
BCHE	butyrylcholinesterase	−2.55E+ 00	1.00E-02	0.2471753
ATP6V1D	**ATPase H+ transporting V1 subunit D**	**−2.55E+ 00**	**1.54E-04**	**0.1028588**
ENST00000390323	NA	−2.55E+ 00	2.42E-02	0.2937808
CCR2	C-C motif chemokine receptor 2	−2.54E+ 00	9.37E-03	0.24523
ENST00000479981	NA	−2.54E+ 00	9.43E-03	0.24523
CAST	**calpastatin**	**−2.54E+ 00**	**1.02E-03**	**0.1525447**
ASPN	asporin	−2.52E+ 00	2.94E-02	0.3049408
HBB	hemoglobin subunit beta	−2.52E+ 00	2.08E-02	0.2862875
ENST00000390247	NA	−2.51E+ 00	2.16E-02	0.2871745
SULF1	sulfatase 1	−2.50E+ 00	1.80E-02	0.2805948
EPYC	epiphycan	−2.49E+ 00	1.28E-02	0.2588936
FGFBP1	fibroblast growth factor binding protein 1	−2.49E+ 00	1.03E-02	0.2499163
LRRC15	leucine rich repeat containing 15	−2.48E+ 00	4.14E-02	0.3321649
DOCK11	dedicator of cytokinesis 11	−2.45E+ 00	5.13E-04	0.1261184
OOEP	oocyte expressed protein	−2.45E+ 00	4.23E-03	0.2013983
ROR2	**receptor tyrosine kinase like orphan receptor 2**	**−1.84E+ 00**	**2.83E-05**	**0.0506967**

mRNAs selected for further validation are shown in bold.

most downregulated genes are summarized in Tables 4 and 5, respectively. The comparison of patient groups according to PARP levels and treatment responses are shown in Figure 1, a-b.

GREB1 and ROR2 are Significantly Differentially Expressed in Ovarian Cancer Tumor Samples Based on Platinum Sensitivity and PARP Levels

Fourteen differentially expressed mRNAs (namely: *ROR2, CAST, ATP6V1D, GUCY1A3, TMOD1, MYCN, DLK1, PLEKHG4B, GREB1, B4GALNT4, SLC35F3, PTCH2, TNNC1, BNC1*) from the microarray analysis were selected for validation by qRT-PCR in the cohort of 53 ovarian cancer patients, based on fold change, q-value, signal intensity and previous literature (Table 2). Two genes, namely *ROR2* and *GREB1*, were significantly differentially expressed in high PARP/platinum-sensitive vs. low PARP/platinum-resistant groups (P = .02 and 0.002, respectively) (Figure 2, a-b). *ROR2* was downregulated in microarray data in high PARP/platinum-sensitive tumor samples and the same result was obtained by qRT-PCR analysis in the validation cohort (n = 53). A trend towards statistically significant difference between the platinum sensitive and resistant groups regardless of PARP levels was observed (P = .058) (Figure 2a). The comparisons described above were based on treatment response as a significant clinical feature and issue of interest. In addition, previously determined PARP levels were taken into account to further investigate tumors from the BRCAness profile point of view. The platinum sensitive and high PARP level cohort is considered a group of better prognosis befitting with the BRCAness profile, whereas the platinum resistant and low PARP level a group with poorer prognosis.

On the other hand, *GREB1* was upregulated in microarray data in high PARP/platinum-sensitive tumor samples, and this result was validated by qRT-PCR (Figure 2b). Furthermore, GREB1 also showed a trend of overexpression in platinum sensitive samples, regardless of PARP level (P = .26).

GREB1 Expression Defines Platinum Sensitivity and Correlates with Longer PFS in Ovarian Cancer

Furthermore, we investigated whether *GREB1* expression affects PFS in ovarian cancer patients (n = 53). High *GREB1* expression was significantly associated with longer PFS as shown in Figure 2c (P = .019 in log-rank test). In Kaplan–Meier database search, which included 1465 ovarian cancer patients, a similar result was shown associating high GREB1 expression with better PFS (P = 0,014 in log-rank test), as shown in Figure 2d. In addition to Kaplan–Meier plotter database and Oncomine (website links are reported in statistical analysis section) were searched for the *ROR2* and *GREB1* genes. No similar results in expression correlation were found, however, no similar study settings were found (HGSC, treatment response comparison).

High ROR2 Expression in LowPARP/Platinum Resistant Ovarian Cancer Samples Correlated with Higher Wnt5a, STAT3 and NF-kB levels

Previous data have shown no association with ROR2 expression and relapse-free survival [25] in ovarian cancer. However, ROR2 and ROR1 expression is increased in cisplatin resistant A2780 cell line compared to parental cells ([18]), and silencing their ligand Wnt5a in serous adenocarcinoma OVCAR3 cell line had greater effect in inhibiting cell migration and invasion than silencing either ROR alone [25]. Since *ROR2* was significantly upregulated in low PARP/platinum-resistant patient samples in our microarray data, we decided

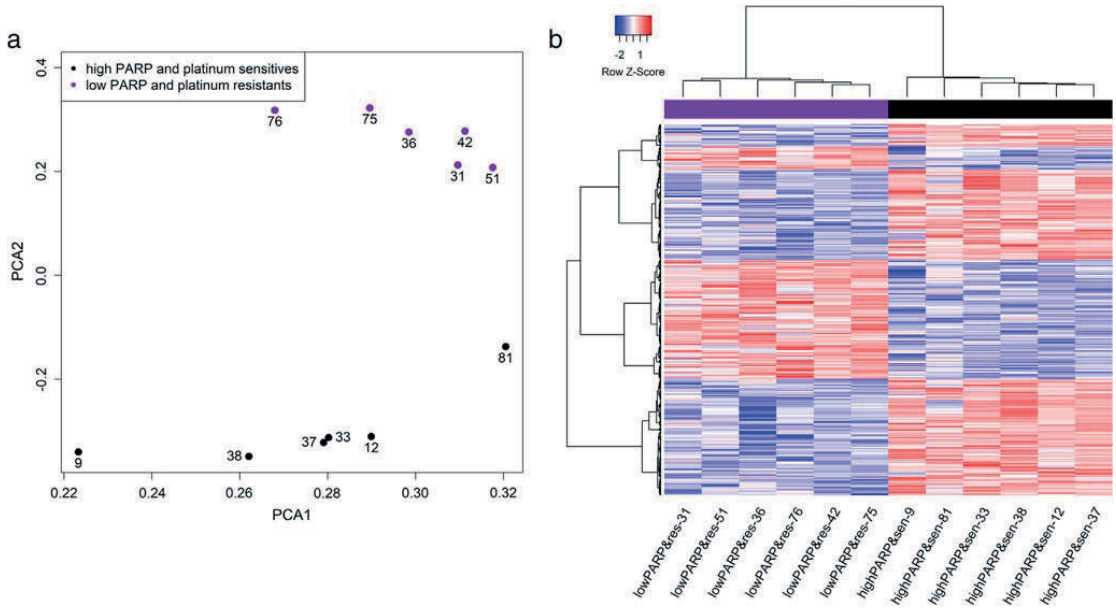

Figure 1. a. Scatterplot showing location of samples of high PARP and platinum sensitive and low PARP and platinum resistant in patients along the first two principal components (PC1 and PC2). Platinum sensitive and high PARP level samples are represented by black and low PARP and platinum resistant samples by purple dots.b. Unsupervised clustering of the clinical samples shows differential expression patterns in patients with high PARP levels and platinum sensitivity compared to patients with low PARP levels and platinum resistant.

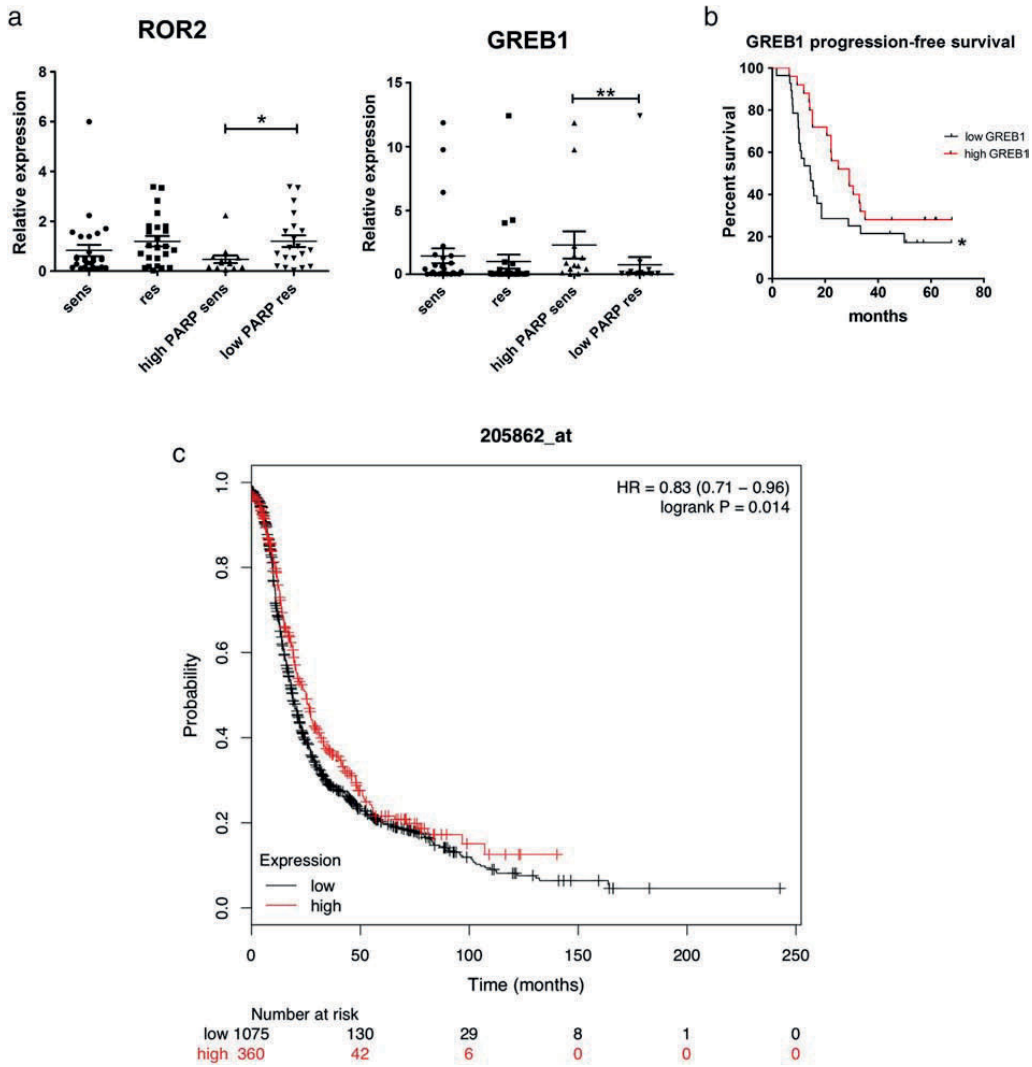

Figure 2. a. Expression of ROR2 and GREB1 in platinum sensitive (n = 38) vs resistant (n = 15) samples as well as high PARP level and platinum sensitive (n = 17) vs low PARP level and platinum resistant (n = 14) samples according to qRT-PCR (P = .02 and 0.002 respectively). The graphs show mean ± SEM.b. Kaplan–Meier analysis of progression free survival (PFS) according to median level of GREB 1 concentration (log rank P = .019). Vertical lines represent censored patients.c. Kaplan–Meier analysis of progression free survival (PFS) according to median level of GREB 1 concentration (log-rank P = .014) from Kaplan–Meier plotter database.

to investigate the expression levels of Wnt5a, ROR1 and ROR2 proteins by Western blot in a subcohort of samples described in Table 3. As shown in Figure 3a-b, higher expression levels of Wnt5a, ROR1 and ROR2 proteins were found in low PARP/platinum-resistant group compared to high PARP/platinum-sensitive group. Moreover, upregulation of downstream signaling mediators such as pSTAT3 (Y705) and NF-kB was also observed in tumor lysates from low PARP/platinum resistant group. Noteworthy, patient samples with high levels of ROR1 and ROR2 from low PARP/platinum-resistant group showed higher pSTAT3 (Y705) levels (Figure 3a), indicating that STAT3 could mediate signaling downstream ROR1 and ROR2. Database search (Oncomine, Kaplan–Meier plotter; website links are reported in statistical analysis section) revealed no additional information regarding ROR in therapy response and ovarian cancer setting.

ROR2 and GREB1 Show Differential Expression in Cisplatin Resistant Ovarian Cancer Cell Line Model

The human epithelial ovarian cancer cell line A2780 and its cisplatin resistant model A2780cis were selected to investigate the expression levels of ROR2 and GREB1. We confirmed the chemoresistant phenotype of A2780cis cells compared to A2780 parental cells by CTG assay and found significant increase of EC50 to cisplatin treatment for A2780cis cells (Figure 4a). The expression of both ROR2 and GREB1 mRNA level was investigated in A2780 and

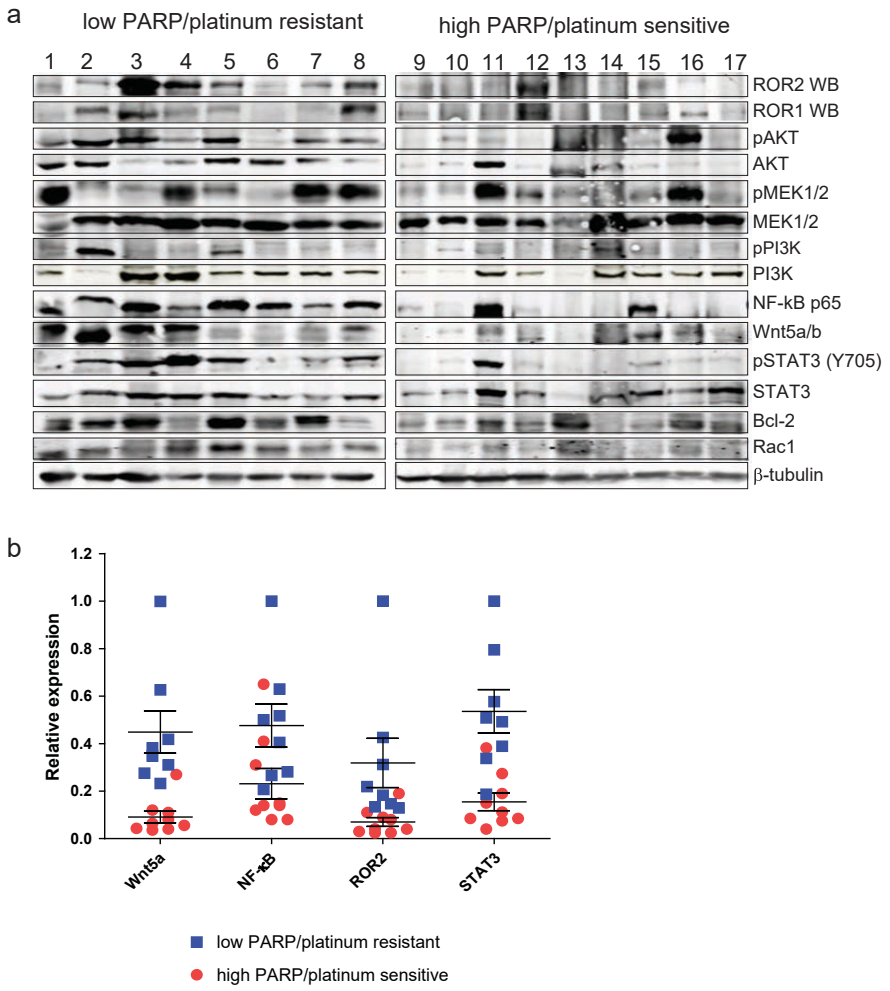

Figure 3. Wnt5a/ROR2 expression is increased in lysates from platinum resistant tumors. A. Western blot analysis of lysates from platinum resistant vs. platinum sensitive patient samples with the indicated primary antibodies. B. Quantification of protein expression for Wnt5a, ROR2, STAT3 and NF-kB based on β-tubulin levels. Horizontal lines represent the average and errors bars are SEM (standard error of mean). After normalization, samples were quantified based on the highest expression level of all samples.

A2780cis cells (Figure 4b and c). We observed ROR2 mRNA upregulation in platinum resistant cell line A2780cis compared to platinum sensitive A2780 cells (P = .0046), as previously shown ([18]), whereas GREB1 mRNA level was downregulated in A2780cis compared to A2780 parental cells (P = .0012). Furthermore, A2780cis cells have increased protein expression levels of ROR2 and Wnt5a compared with the parental A2780 cells as shown by Western blotting of cytoplasmic cell lysates (Figure 4d). In addition, we detected higher nuclear expression levels for STAT3 and NF-kB in chemoresistant A2780cis compared to parental A2780 cells (Figure 4d). Thus, our microarray data from patient samples are validated in chemoresistant ovarian cancer cell line and investigations are ongoing to decipher the molecular mechanisms employed by ROR2 and GREB1 for their differential expression associated with cisplatin resistance.

Discussion

Due to its poor prognosis, identification of new therapeutic approaches is highly needed in ovarian cancer. Although approximately 50% of HGSC tumors with HR defects may benefit from PARP inhibitors, beyond this, identification of other commonly deregulated pathways could provide opportunities for better therapeutic interventions. As such, our goal was to examine gene and protein expression in a HGSC tumor sample cohort defined by response to platinum therapy and the level of PARP expression. Patients were carefully stratified based on their PARP levels and platinum responsiveness as previously indicated [20] and monitored for relatively long time, up to 50 months to ensure a comprehensive analysis of their disease progression.

Two functionally unrelated genes, *GREB1* and *ROR2* were identified in our analysis as significantly differentially expressed

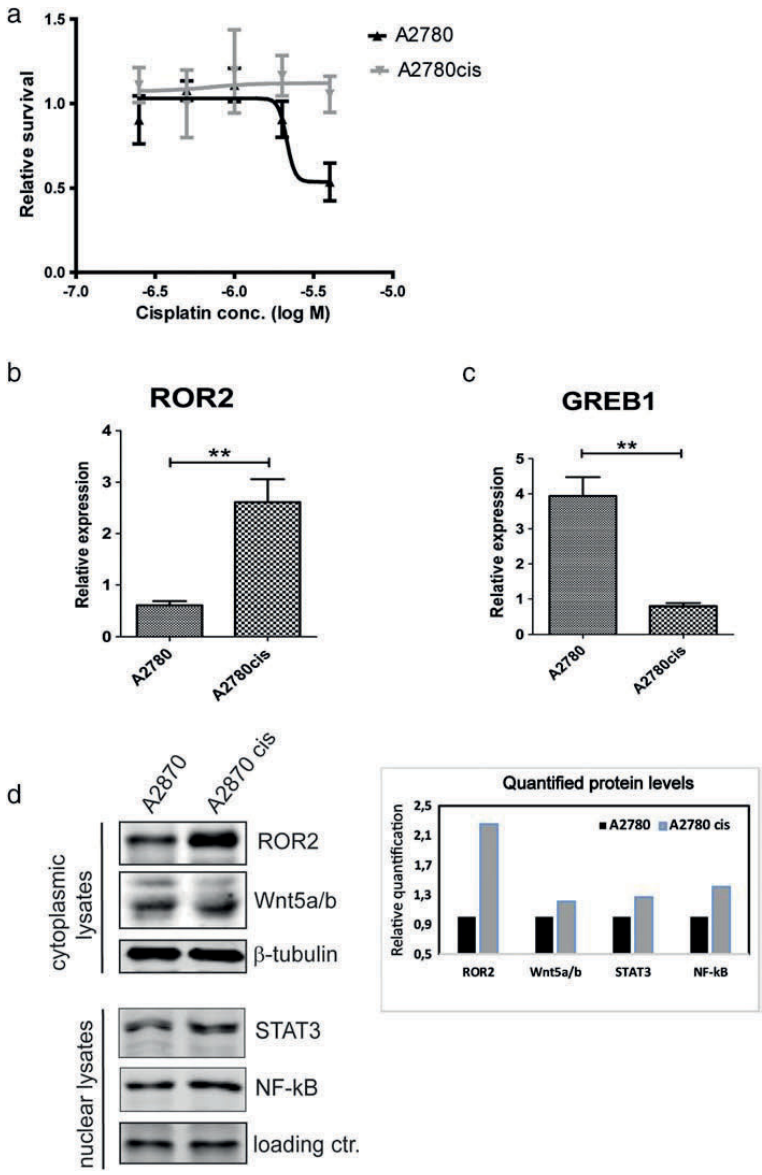

Figure 4. a. Cisplatin sensitivity testing of A2780 and A2780cis cell lines. Cells were incubated as five replicates for 3 days with increased concentrations of cisplatin as indicated and cell-viability was measured by CTG assay. EC50 was calculated using Graph Prism software and shown as approximate values.b. ROR2 mRNA expression in platinum sensitive and resistant cell lines A2780/A2780cis ($P = .0046$). The graphs show mean ± SEM.c. GREB1 mRNA expression in platinum sensitive and resistant cell lines A2780/A2780cis ($P = .0012$. The graphs show mean ± SEM.d. Protein expression levels for ROR2, Wnt5a, NF-kB and STAT3 in the cytoplasmic and nuclear cell lysates of A2780 and A2780cis cell lines. Protein quantification was done using Oddyssey Licor software and normalized against the loading control.

between high PARP/platinum-sensitive and low PARP/platinum-resistant groups.

While initially sequenced from brain tissue, human *GREB1* is highly expressed in normal and neoplastic ovarian tissue and in several other hormone-responsive tissues such as breast, uterine and prostate [26–29]. *GREB1*, along with *CCND1* and *MYC*, are common transcription targets for E2 (17β-estradiol)-mediated proliferative responses, via ESR1 (estrogen receptor one) engagement [28]. Estrogen receptor positive ($ESR1^{+}$) breast cancers usually express GREB1, whereas in ovarian cancer, its expression could be detected in both, $ESR1^{+}$ or estrogen receptor negative ($ESR1^{-}$) tumors. ESR1-independent expression of GREB1 may indicate the existence of

other signaling pathways for estrogen-promoting growth in the absence of E2 and/or ESR1, underlining differences in E2-responsiveness between breast and ovarian cancer that play an important role in response to antiestrogenic therapies [30]. *GREB1* was upregulated in all EOC tumors and was suggested to have potential biomarker role in ovarian cancer [29]. *GREB1* was upregulated in our microarray data in high PARP/platinum-sensitive patients, and high *GREB1* expression was associated with longer PFS, suggesting a prognostic value for *GREB1* in ovarian cancer.

It has been demonstrated that *GREB*1 knockdown inhibits proliferation of ovarian cancer cell lines and consequently, prolongs survival in an orthotopic mouse model [28]. Also, hypomethylation at specific CpG site associated with *GREB1* has been associated with longer PFS in ovarian cancer in a DNA methylation study designed to investigate epigenetic modifications [31]. Interestingly, loss of GREB1 has been linked to tamoxifen resistance in breast cancer due to loss of sensitivity to endocrine agents in general, underlining its important role in endocrine resistance [32–33]. Although endocrine therapy has overall limited efficacy in ovarian cancer patients, more research is needed to assess whether GREB1 is associated with antiestrogen sensitivity in this cancer. In view of this previous data, our results are in accordance with these findings reaffirming the understanding that *GREB1* is highly expressed in high PARP/platinum-sensitive patient group that has been associated with a positive outcome befitting with the BRCAness profile [10]. Our data show for the first time that *GREB1* could have prognostic value in ovarian cancer and warrants further investigation, especially associated with response to hormone-based therapy.

ROR2 belongs to the ROR receptor family (*ROR1* and *ROR2*) from the non-canonical Wnt pathway [17]. ROR1 and ROR2 form heterodimers in response to Wnt5a, which leads to RhoA/Rac1 activation and increases migration and invasion properties of cancer cells [34]. Recent studies have demonstrated that upregulation of *ROR2* and its ligand Wnt5a in EOC regulates EMT and correlates with worse prognosis [35]. *ROR1* and *ROR2* regulate migration and invasion of ovarian cancer cells and more importantly, their expression was increased in platinum resistant A2780 ovarian cancer cell line compared to parental cells ([18]). TCGA data analysis of over 500 ovarian tumor samples identified high expression of Wnt5a and Wnt5a protein was found prevalent in ascites samples of ovarian cancer patients [36]. In our study, *ROR2* gene expression was significantly upregulated in low PARP/platinum-resistant vs. high PARP/platinum-sensitive patient samples. We also found higher protein levels of ROR2, ROR1 and Wnt5a ligand in lysates of platinum-resistant tumors (Figure 3, a-b), confirming our gene expression analysis. Interestingly, higher expression levels of NF-κB and pSTAT3 (Y705) proteins were also noted in platinum-resistant tumor lysate with high ROR1 and ROR2 levels, indicating that STAT3 could be downstream mediator of ROR signaling in ovarian cancer. These results showing Wnt5a-ROR2 and STAT3/NF-kB signaling pathway in clinical ovarian tumor samples are novel findings. A direct link between ROR1 and STAT3 expression has been demonstrated previously in leukemia, showing that STAT3 promoter harbors two ROR1 binding sites [37]. Moreover, previous studies have shown that Wnt5a is involved in cancer multidrug resistance (MDR)[36]. High Wnt5a expression levels in ovarian cancer cell lines correlated with lower chemosensitivity to paclitaxel, oxaliplatin, 5-fluorouracil, epirubicin and etoposide ([38,39]. Furthermore, upregulation of Wnt5a and ROR2 was detected in colon cancer cells resistant to butyrate, a histone deacetylase inhibitor (HDACi)[40], along with activation of AKT/PKB (protein kinase B) signaling pathway. Wnt5a orchestrates multiple signaling networks involved in chronic inflammation and carcinogenesis, and crosstalk with STAT3 and NF-κB pathways have been previously documented [41]. Thus, upregulation of Wnt5a, ROR1 and ROR2 signaling pathways could be common mechanisms in ovarian cancer chemoresistance and could serve as putative biomarkers. ROR1 targeted therapies have shown promising results in preclinical and clinical models, with anti-ROR1 monoclonal antibody cirmtuzumab being efficient not only in leukemia, but also in ovarian cancer [42], indicative of high therapeutic potential for targeting these receptors.

Our mRNA expression data from patient samples were validated using a cisplatin resistant cell line model A2780cis and we found that upregulation of ROR2 at the mRNA and protein levels is associated with cisplatin resistance. Moreover, GREB1 mRNA level was downregulated in A2780cis compared to A2780 parental cells. Furthermore, we observed higher expression levels for Wnt5a, STAT3 and NF-kB proteins in chemoresistant A2780cis cells compared to parental cells, in support of our results from patient samples (Figure 3).

In conclusion, we found the expression of ROR2 and GREB1 to be associated with treatment response in HGSC. The association between Wnt5a/ROR2 expression and development of platinum resistance reported herein suggests that the Wnt5a/ROR2 pathway is potentially actionable for possible modulation of chemoresistance. Because silencing *ROR1* and *ROR2* restores the chemosensitivity of carboplatin-resistant ovarian cancer cells [18], a combination of ROR antagonists and chemotherapeutic agents may offer a promising treatment option. Also, our findings regarding *GREB1* expression in highPARP/platinum-sensitive patients should reinforce the interest in this gene for future investigations.

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Disclosure

The authors declare no conflicts of interest.

References

[1] Nowak M, Głowacka E, Lewkowicz P, Banasik M, Szyłło K, Zimna K, Bednarska K, and Klink M (2018). Sub-optimal primary surgery leads to unfavorable immunological changes in ovarian cancer patients. *Immunobiology* **223**, 1–7. https://doi.org/10.1016/j.imbio.2017.10.021.

[2] Heintz APM, Odicino F, Maisonneuve P, Quinn MA, Benedet JL, Creasman WT, Ngan HYS, Pecorelli S, and Beller U (2006). Carcinoma of the ovary. FIGO 26th Annual Report on the Results of Treatment in Gynecological Cancer. *Int J Gynaecol Obstet* **95**(Suppl 1), S161–S192. https://doi.org/10.1016/S0020-7292(06)60033-7.

[3] Matsuda A and Katanoda K (2014). Five-year Relative Survival Rate of Ovarian Cancer in the USA, Europe and Japan. *Jpn J Clin Oncol* **44**, 196. https://doi.org/10.1093/jjco/hyu007.

[4] Levanon K, Crum C, and Drapkin R (2008). New insights into the pathogenesis of serous ovarian cancer and its clinical impact. *J Clin Oncol Off J Am Soc Clin Oncol* **26**, 5284–5293. https://doi.org/10.1200/JCO.2008.18.1107.

[5] Cancer Genome Atlas Research Network (2011). Integrated genomic analyses of ovarian carcinoma. *Nature* **474**, 609–615. https://doi.org/10.1038/nature10166.

[6] Bast RC (2011). Molecular approaches to personalizing management of ovarian cancer. *Ann Oncol* **22**(Suppl 8), viii5–viii15. https://doi.org/10.1093/annonc/mdr516.

[7] Davar D, Beumer JH, Hamieh L, and Tawbi H (2012). Role of PARP inhibitors in cancer biology and therapy. *Curr Med Chem* **19**, 3907–3921.

[8] Fang B (2014). Development of synthetic lethality anticancer therapeutics. *J Med Chem* **57**, 7859–7873. https://doi.org/10.1021/jm500415t.

[9] Bast RC and Mills GB (2010). Personalizing therapy for ovarian cancer: BRCAness and beyond. *J Clin Oncol Off J Am Soc Clin Oncol* **28**, 3545–3548. https://doi.org/10.1200/JCO.2010.28.5791.

[10] Konstantinopoulos PA, Spentzos D, Karlan BY, Taniguchi T, Fountzilas E, Francoeur N, Levine DA, and Cannistra SA (2010). Gene expression profile of BRCAness that correlates with responsiveness to chemotherapy and with outcome in patients with epithelial ovarian cancer. *J Clin Oncol Off J Am Soc Clin Oncol* **28**, 3555–3561. https://doi.org/10.1200/JCO.2009.27.5719.

[11] Ramos P and Bentires-Alj M (2015). Mechanism-based cancer therapy: resistance to therapy, therapy for resistance. *Oncogene* **34**, 3617–3626. https://doi.org/10.1038/onc.2014.314.

[12] Liu X, Gao Y, Lu Y, Zhang J, Li L, and Yin F (2015). Oncogenes associated with drug resistance in ovarian cancer. *J Cancer Res Clin Oncol* **141**, 381–395. https://doi.org/10.1007/s00432-014-1765-5.

[13] Yin F, Liu X, Li D, Wang Q, Zhang W, and Li L (2013). Tumor suppressor genes associated with drug resistance in ovarian cancer (Review). *Oncol Rep* **30**, 3–10. https://doi.org/10.3892/or.2013.2446.

[14] Miow QH, Tan TZ, Ye J, Lau JA, Yokomizo T, Thiery J-P, and Mori S (2015). Epithelial-mesenchymal status renders differential responses to cisplatin in ovarian cancer. *Oncogene* **34**, 1899–1907. https://doi.org/10.1038/onc.2014.136.

[15] Baarsma HA, Königshoff M, and Gosens R (2013). The WNT signaling pathway from ligand secretion to gene transcription: molecular mechanisms and pharmacological targets. *Pharmacol Ther* **138**, 66–83. https://doi.org/10.1016/j.pharmthera.2013.01.002.

[16] Ford CE, Henry C, Llamosas E, Djordjevic A, and Hacker N (2016). Wnt signalling in gynaecological cancers: A future target for personalised medicine? *Gynecol Oncol* **140**, 345–351. https://doi.org/10.1016/j.ygyno.2015.09.085.

[17] Endo M, Nishita M, Fujii M, and Minami Y (2015). Insight into the role of Wnt5a-induced signaling in normal and cancer cells. *Int Rev Cell Mol Biol* **314**, 117–148. https://doi.org/10.1016/bs.ircmb.2014.10.003.

[18] Henry CE, Llamosas E, Djordjevic A, Hacker NF, and Ford CE (2016). Migration and invasion is inhibited by silencing ROR1 and ROR2 in chemoresistant ovarian cancer. *Oncogene* **5**, e226. https://doi.org/10.1038/oncsis.2016.32.

[19] National Cancer Policy ForumBoard on Health Care ServicesHealth and Medicine DivisionNational Academies of Sciences, Engineering, and Medicine (2017.). The Drug Development Paradigm in Oncology: Proceedings of a Workshop, The National Academies Collection: Reports funded by National Institutes of Health. Washington (DC): National Academies Press (US); 2017.

[20] Veskimäe K, Staff S, Grönholm A, Pesu M, Laaksonen M, Nykter M, Isola J, and Mäenpää J (2016). Assessment of PARP protein expression in epithelial ovarian cancer by ELISA pharmacodynamic assay and immunohistochemistry. *Tumour Biol* **37**, 11991–11999. https://doi.org/10.1007/s13277-016-5062-6.

[21] Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, and Gentry J, et al (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* **5**, R80. https://doi.org/10.1186/gb-2004-5-10-r80.

[22] Bolstad BM, Irizarry RA, Astrand M, and Speed TP (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19**, 185–193.

[23] Smyth GK (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3**3. https://doi.org/10.2202/1544-6115.1027.

[24] Mardia KV, Li Q, and Hainsworth TJ (1992). On the Penrose hypothesis on fingerprint patterns. *IMA J Math Appl Med Biol* **9**, 289–294.

[25] Henry C, Llamosas E, Knipprath-Meszaros A, Schoetzau A, Obermann E, Fuenfschilling M, Caduff R, Fink D, Hacker N, and Ward R, et al (2015). Targeting the ROR1 and ROR2 receptors in epithelial ovarian cancer inhibits cell migration and invasion. *Oncotarget* **6**, 40310–40326. https://doi.org/10.18632/oncotarget.5643.

[26] Hnatyszyn HJ, Liu M, Hilger A, Herbert L, Gomez-Fernandez CR, Jorda M, Thomas D, Rae JM, El-Ashry D, and Lippman ME (2010). Correlation of GREB1 mRNA with protein expression in breast cancer: validation of a novel GREB1 monoclonal antibody. *Breast Cancer Res Treat* **122**, 371–380. https://doi.org/10.1007/s10549-009-0584-x.

[27] Fung JN, Holdsworth-Carson SJ, Sapkota Y, Zhao ZZ, Jones L, Girling JE, Paiva P, Healey M, Nyholt DR, and Rogers PAW, et al (2015). Functional evaluation of genetic variants associated with endometriosis near GREB1. *Hum Reprod* **30**, 1263–1275. https://doi.org/10.1093/humrep/dev051.

[28] Pellegrini C, Gori I, Achtari C, Hornung D, Chardonnens E, Wunder D, Fiche M, and Canny GO (2012). The expression of estrogen receptors as well as GREB1, c-MYC, and cyclin D1, estrogen-regulated genes implicated in proliferation, is increased in peritoneal endometriosis. *Fertil Steril* **98**, 1200–1208. https://doi.org/10.1016/j.fertnstert.2012.06.056.

[29] Hodgkinson KM (2016). The role of steroid hormones, GREB1, and reproductive status in ovarian cancer progression. Ottawa, Ontario, Canada: Department of Cellular and Molecular Medicine University of Ottawa; 2016 .

[30] Schaner ME, Ross DT, Ciaravino G, Sorlie T, Troyanskaya O, Diehn M, Wang YC, Duran GE, Sikic TL, and Caldeira S, et al (2003). Gene expression patterns in ovarian carcinomas. *Mol Biol Cell* **14**, 4376–4386. https://doi.org/10.1091/mbc.E03-05-0279.

[31] Bauerschlag DO, Ammerpohl O, Bräutigam K, Schem C, Lin Q, Weigel MT, Hilpert F, Arnold N, Maass N, and Meinhold-Heerlein I, et al (2011). Progression-free survival in ovarian cancer is reflected in epigenetic DNA methylation profiles. *Oncology* **80**, 12–20. https://doi.org/10.1159/000327746.

[32] Mohammed H, D'Santos C, Serandour AA, Ali HR, Brown GD, Atkins A, Rueda OM, Holmes KA, Theodorou V, and Robinson JLL, et al (2013). Endogenous purification reveals GREB1 as a key estrogen receptor regulatory factor. *Cell Rep* **3**, 342–349. https://doi.org/10.1016/j.celrep.2013.01.010.

[33] Wu Y, Zhang Z, Cenciarini ME, Proietti CJ, Amasino M, Hong T, Yang M, Liao Y, Chiang H-C, and Kaklamani VG, et al (2018). Tamoxifen Resistance in Breast Cancer Is Regulated by the EZH2-ERα-GREB1 Transcriptional Axis. *Cancer Res* **78**, 671–684. https://doi.org/10.1158/0008-5472.CAN-17-1327.

[34] Yu J, Chen L, Cui B, Widhopf GF, Shen Z, Wu R, Zhang L, Zhang S, Briggs SP, and Kipps TJ (2016). Wnt5a induces ROR1/ROR2 heterooligomerization to enhance leukemia chemotaxis and proliferation. *J Clin Invest* **126**, 585–598. https://doi.org/10.1172/JCI83535.

[35] Ford CE, Punnia-Moorthy G, Henry CE, Llamosas E, Nixdorf S, Olivier J, Caduff R, Ward RL, and Heinzelmann-Schwarz V (2014). The non-canonical Wnt ligand, Wnt5a, is upregulated and associated with epithelial to mesenchymal transition in epithelial ovarian cancer. *Gynecol Oncol* **134**, 338–345. https://doi.org/10.1016/j.ygyno.2014.06.004.

[36] Asem M, Buechler S, Wates R, Miller D, and Stack M (2016). Wnt5a Signaling in Cancer. *Cancer* **8**, 79. https://doi.org/10.3390/cancers8090079.

[37] Li P, Harris D, Liu Z, Liu J, Keating M, and Estrov Z (2010). Stat3 activates the receptor tyrosine kinase like orphan receptor-1 gene in chronic lymphocytic leukemia cells. *PLoS One* **5**e11859. https://doi.org/10.1371/journal.pone.0011859.

[38] Peng C, Zhang X, Yu H, Wu D, and Zheng J (2011). Wnt5a as a predictor in poor clinical outcome of patients and a mediator in chemoresistance of ovarian cancer. *Int J Gynecol Cancer* **21**, 280–288. https://doi.org/10.1097/IGC.0b013e31820aaadb.

[39] Varma RR, Hector SM, Clark K, Greco WR, Hawthorn L, and Pendyala L (2005). Gene expression profiling of a clonal isolate of oxaliplatin-resistant ovarian carcinoma cell line A2780/C10. *Oncol Rep* **14**, 925–932.

[40] Bordonaro M, Tewari S, Cicco CE, Atamna W, and Lazarova DL (2011). A switch from canonical to noncanonical Wnt signaling mediates drug resistance in colon cancer cells. *PLoS One* **6**e27308. https://doi.org/10.1371/journal.pone.0027308.

[41] Katoh (2009). Transcriptional mechanisms of WNT5A based on NF-κB, Hedgehog, TGFβ, and Notch signaling cascades. *Int J Mol Med* **23**. https://doi.org/10.3892/ijmm_00000190.

[42] Choi MY, Widhopf GF, Wu CCN, Cui B, Lao F, Sadarangani A, Cavagnaro J, Prussak C, Carson DA, and Jamieson C, et al (2015). Pre-clinical Specificity and Safety of UC-961, a First-In-Class Monoclonal Antibody Targeting ROR1. *Clin Lymphoma Myeloma Leuk* **15**(Suppl), S167–S169. https://doi.org/10.1016/j.clml.2015.02.010.